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THERMODYNAMIC PROPERTIES OF SOLUTIONS

OF LONG-CHAIN COMPOUNDS*

BY

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CONTENTS

INTRODUCTION

The great and rapidly increasing industrial importance of systems containing long-chain compounds needs no emphasis. In spite of their importance, most of the progress that has been made in correlating the properties of these systems with their compositions has been made empirically. Much evidence has accumulated to show that many of the theoretical relationships applicable to systems containing only small molecules cannot be applied, without substantial modification, to systems containing giant chain molecules. This has been especially true with regard to solutions. There has been no quantitative theory of the thermodynamic properties of these solutions—those properties (vapor pressures, osmotic pressures, freezing points, boiling points, solubilities, formation of gels and absorption of solvents by them, etc.) which depend directly on the activities of the component substances.

In the present paper[1] this lack is remedied. Using well-known statistical methods, equations for the activities are derived and shown to be in good agreement with the considerable amount of experimental data in the literature which is suitable for testing them. One can therefore now proceed with confidence to apply these equations to other systems than those tested and to all properties (such as those mentioned above) depending on the activities.

The equations derived are also applicable to solutions containing only molecules of small molecular weight. They furnish a means of calculating the magnitude of the effect of the volume, shape and flexibility of the component molecules on the entropy or randomness contribution to the activities—effects which are usually neglected for want of a satisfactory theory, but which are often far from negligible.

In recent years it has become increasingly evident, largely as a result of extensive studies of the thermodynamic properties of solutions of chain molecules by Meyer and his collaborators, [2-9] that the large deviations from "ideal" behavior shown by such solutions—especially when the

[1]An outline of the material herein was presented before the Division of Physical and Inorganic Chemistry of the American Chemical Society at Atlantic City, September 11, 1941. A preliminary report, presented at the W. D. Bancroft Colloid Symposium, Ithaca, New York, on June 20, 1941, has just been published. **Huggins, M. L.** Jour. Phys. Chem. **46**: 151. 1942. A brief note, dealing with the application of the results to osmotic pressure data, has also been published. **Huggins, M. L.** Jour. Chem., Phys. **9**: 440. 1941.

[2] **Meyer, K. H., & Lühdemann, R.** Helv. Chim. Acta. **18**: 307. 1935.

[3] **Boissonas, C. G.** Helv. Chim. Acta. **20**: 768. 1937.

[4] **Boissonas, C. G., & Meyer, K. H.** Helv. Chim. Acta. **20**: 783. 1937; Z. physik. Chem. **B 40**: 108. 1938.

[5] **Meyer, K. H.** Z. physik. Chem. **B 44**: 383. 1939; Helv. Chim. Acta. **23**: 1063. 1940.

[6] **Meyer, K. H., Wolff, E., & Boissonas, C. G.** Helv. Chim. Acta. **23**: 430. 1940.

[7] **Wolff, E.** Helv. Chim. Acta. **23**: 439. 1940.

[8] **Hagger, O., & van der Wyk, A. J. A.** Helv. Chim. Acta. **23**: 484. 1940.

[9] **Meyer, K. H., & van der Wyk, A. J. A.** Helv. Chim. Acta. **23**: 485. 1940.

chains are very long—are due to an entropy effect rather than to an energy effect or to solvation. Meyer has discussed the situation very ably from a qualitative standpoint, but no quantitative theoretical treatment has yet been published. Fowler and Rushbrooke[10] and Chang,[11,12] it is true, have made a beginning in this direction, treating statistically the much simpler case of a solution of diatomic dumbbell molecules in spherical monatomic molecules.[*] Very recently, also, Flory[13] has announced that he has carried through a statistical treatment along the same lines as the present one, with results which are at least approximately the same.

In this paper, equations will be derived for the partial molal entropies of dilution in idealized solutions of chain compounds and for the activities and various other related quantities. The applicability of these equations to actual solutions will be discussed from a theoretical standpoint and tested by comparison with experimental data.

DERIVATION OF THE ACTIVITY EQUATIONS

We consider first a hypothetical solution consisting of N_1 spherical molecules and N_2 chain molecules, each of the latter consisting (FIGURE 1) of n submolecules of the same size and shape as each type 1 molecule. Similarity in shape is probably unimportant, as with simple solutions. We assume that there is no volume change on mixing and that the intermolecular energy interactions are not such as to interfere with the randomness which would result if the heat of mixing were zero.

Following the procedure applied by Fowler and Rushbrooke[10] to solutions of diatomic molecules in monatomic molecules, we distribute the type 1 molecules and type 2 submolecules among

$$N = N_1 + nN_2 \tag{1}$$

sites, first adding the type 2 molecules, one submolecule at a time, then the type 1 molecules. We count the number of different ways in which each can be added and then multiply these numbers together to obtain the total number of configurations. From the total number of *different* configurations possible, we can proceed by well-known methods to the desired equations for the activities.

[10] **Fowler, R. H., & Rushbrooke, G. S.**   Trans. Faraday Soc. **33**: 1272. 1937.
[11] **Chang, T. S.**   Proc. Cambridge Phil. Soc. **35**: 265. 1939.
[12] **Fowler, R. H., & Guggenheim, E. A.** "Statistical Thermodynamics." University Press, Cambridge, England. 1939.
[*] Hildebrand see Ref. 22 has dealt with the special case of mixtures of rodlike molecules of different length
[13] **Flory, P. J.**   Jour. Chem. Phys. **9**: 660. 1941; **10**: 51. 1942.
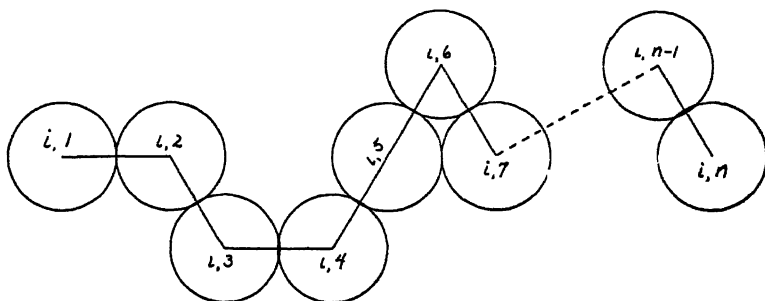
FIGURE 1. Illustrating the designation of submolecules in the i-th type 2 molecule (first model).

Let ν_{ij} be the number of alternative sites available for the j-th submolecule of the i-th type 2 molecule. Obviously,

$$\nu_{1,1} = N \tag{2}$$

and

$$\nu_{1,2} = z, \tag{3}$$

where z is the coördination number, the number of neighbor sites immediately surrounding each site. Also,

$$\nu_{1,3} = y, \tag{4}$$

where

$$y = z - 1, \tag{5}$$

assuming all of the $z - 1$ unoccupied sites around submolecule 1, 2 to be equally probable.

The average numbers of alternatives for the 4th and subsequent submolecules are each decreased by a factor to allow for the fact that an otherwise available site may be already occupied. This factor will be discussed below.

In general, for the first submolecule of the i-th type 2 molecule,

$$\nu_{i,1} = N(1 - f_{i,1})(1 - f_{i,1}^z), \tag{6}$$

the last factor expressing the fact that a first submolecule can not be placed in a site surrounded by z occupied sites. The chance that all z surrounding sites are occupied is $f_{i,1}^z$. For the second submolecule,

$$\nu_{i,2} = \frac{z(1 - f_{i,2})(1 - f_{i,2}^y)}{(1 - f_{i,2}^z)\left(1 - \frac{2(n-1)}{nz}f_{i,2}\right)} \tag{7}$$

The factors in this expression may be explained as follows. We con-

sider a site S_1 and the z sites $S_1, S_2 \ldots S_z$ immediately around it and wish to calculate the average number of alternatives for submolecule $i.2$ if $i.1$ is at S_0.

Let $f_{.2}$ be the fraction of sites already occupied. (See below, following equation (10), however.) Provided there are no limitations regarding the occupation of neighboring sites, the chance that any given site is unoccupied is $(1 - f_{.2})$. Making the approximation that the state of occupation of any one of the sites $S_1 \ldots S_z$ is unaffected by the states of occupation of the neighbors, the sum of the products of the number of unoccupied sites (in the group $S_1 \ldots S_z$) times the chance that there is that number of unoccupied sites is $z(1 - f_{.2})$. To obtain the average number of alternatives, $\nu_{i.2}$, we must divide this by $(1 - f_{.2}^z)$, the probability that not all of the sites $S_1 \ldots S_z$ are occupied, and, unless the chain contains but two submolecules, multiply by $(1 - f_i^y{}_{.2})$, the probability that not all of the sites surrounding S_1 are occupied.

We must also take account of the fact that if S_1, for example, is occupied, the chance that S_0 is occupied by another submolecule in the same chain is much greater than $f_{.2}$. The chance that S_1 is occupied by the first or the last submolecule of a chain is $\dfrac{2}{n} f_{.2}$ and the chance that it is occupied by an intermediate submolecule is $\left(1 - \dfrac{2}{n}\right) f_{.2}$. In the former case, *one* of the z sites surrounding S_1 must also be occupied; in the latter case, *two* of them must also be occupied. One of these sites surrounding S_1 is S_0. The chance that both S_1 and S_0 are occupied by adjacent submolecules in the same chain is

$$\frac{2f_{.2}}{nz} + \frac{2\left(1 - \dfrac{2}{n}\right)f_{.2}}{z} = \frac{2(n-1)f_{.2}}{nz} \tag{8}$$

Since for our calculation, S_0 can not have been previously occupied, we must divide the expression for $\nu_{.2}$, otherwise obtained, by the factor

$$\left(1 - \frac{2(n-1)}{nz} f_{.2}\right).$$

Similar reasoning leads to the following expressions for the average number of alternatives for the j-th submolecule $(2 < j < n)$ and for the last (n-th) submolecule of the i-th molecule.

$$\nu_{i.j} = \frac{y(1 - f_{.j})\,(1 - f_i^y{}_{.j})}{(1 - f_i^y{}_{.j})\left(1 - \dfrac{2(n-1)}{nz} f_{.j}\right)} = \frac{y(1 - f_{.j})}{1 - \dfrac{2(n-1)}{nz} f_{.j}} \tag{9}$$

$$\nu_{,n} = \frac{y^1 - f_{,n}^1}{(1 - f_i^y{}_n)\left(1 - \frac{2(n-1)}{nz}f_{,n}\right)}.$$ 10

The quantities $f_{,,1}$ and $f_{,,2}$ in equations (6) and (7) represent the fractions of the total number of sites which are already occupied. Strictly speaking, however, the quantities $f_{,,}$ and $f_{,n}$ in equations (9) and (10) should each denote the sum of the fraction (f_i) of the sites already occupied by submolecules of *previously placed* molecules and the average probability (f_j) that one of the $S_1 \ldots S_z$ sites is already occupied by a submolecule of *the same* molecule. For the first, we can write

$$f_i = \frac{(i-1)n\ (1-f_j)}{N}.$$ (11)

The second, f_j, is zero for very small values of n but quite rapidly approaches a constant small value as n increases. It is obviously independent of i. The magnitude of the limiting value for f_j depends on z and, in solutions of actual chain molecules, especially on restrictions regarding constant bond angles.

Computations for $z=6$ with an assumed bond angle of 90°, lead to $f_j=0.080$, constant to within about 0.003 for $j>4$. For larger bond angles, f_j should be smaller. With tetrahedral bond angles, in actual solutions, we may guess that f_j has a value of about 0.05.

We can assume with but little error (for our purpose) that f_j has the same constant value, f_0, for all values of j. For small values of n (say, $n<5$),

$$f_0 \approx 0.$$ (12)

For large values of n, (say, $n>8$),

$$f_0 \approx f_{j,\,\text{max.}}$$ (13)

We can thus write

$$f_{i,j} = f_0 + \frac{(i-1)n\ (1-f_0)}{N}$$ (14)

for all values of i and j, with f_0 a small constant.

After all of the type 2 molecules have been placed, there remain N_1 sites among which the type 1 molecules are to be distributed. For the first of these,

$$\nu_1 = N_1;$$ (15)

for the second,

$$\nu_2 = N_1 - 1;$$ (16)

and for the lth,

$$\nu_l = N_1 - (l-1)$$ (17)

The total number of configurations for the system is obtained by multiplying together the ν's for all the type 2 submolecules and the type 1 molecules. To obtain the number ϕ of *different* configurations, we divide this product by N_2 !, by N_1 !, and by σ^{N_2}, where σ, the average symmetry number of the type 2 molecules, is 2 for n equal to 2 or 3 (if both ends of the molecule are alike) and (practically) unity if n is greater than 3. We obtain

$$\phi = \frac{\Pi\nu_{i,j} \cdot \Pi\nu_l}{N_1!\, N_2!\, \sigma^{N_2}} \tag{18}$$

$$\Pi\nu_{.,j} = \prod_{i=1}^{N_2} \frac{Nzy^{n-2}\,(1 - f_{i,j})^n}{\left(1 - \dfrac{2(n-1)}{nz}f_{i,j}\right)^{n-1}} \tag{19}$$

$$\prod_{l=1}^{N_1} \nu_l = N_1! \tag{20}$$

Substituting into equation (18) and taking logarithms, we obtain

$$\ln\phi = N_2\ln\left(\frac{zy^{n-2}}{\sigma}\right) + N_2\ln N - \ln N_2! + n\sum_{i=1}^{N_2}\ln(1 - f_{i,j})$$
$$- (n-1)\sum_{i=1}^{N_2}\ln\left(1 - \frac{2(n-1)}{nz}f_{ij}\right). \tag{21}$$

From Stirling's approximation for the factorials of large numbers,

$$\ln N_2! = N_2\ln N_2 - N_2. \tag{22}$$

Replacing the two summations by integrals and making use of equation (14),

$$\sum_{i=1}^{N_2}\ln(1 - f_{i,j}) = \int_0^{N_2}\ln\left[1 - f_0 - \frac{n(1 - f_0)i}{N}\right]di \tag{23}$$

$$= N_2\ln(1 - f_0) + \int_0^{N_2}\ln\left(1 - \frac{ni}{N}\right)di \tag{24}$$

$$= N_2\ln(1 - f_0) - N_2 - \frac{N_1}{n}\ln N_1 + \frac{N_1}{n}\ln N \tag{25}$$

and, similarly,

$$\sum\ln\left[1 - \frac{2(n-1)f_{i,j}}{nz}\right] = N_2\ln\left[1 - \frac{2(n-1)f_0}{nz}\right] - N_2$$

$$+\left[\frac{z'}{2n}N_1+\left(\frac{z'}{2}-1\right)N_2\right]\ln\left[\frac{N}{N_1+\left(1-\frac{2}{z'}\right)nN_2}\right]. \qquad (26)$$

where

$$z'=\frac{zn}{(n-1)(1-f_0)}-\frac{2f_0}{1-f_0}. \qquad (27)$$

The entropy of mixing, divided by Boltzmann's constant, is given by

$$\frac{\Delta S_M}{k}=\ln\phi-(\ln\phi)_{N_1=0}-(\ln\phi)_{N_2=0}. \qquad (28)$$

(The last term is equal to zero for the present case.) Substituting equations (22), (25) and (26) in equation (21) and performing the calculation indicated in equation (28), we obtain

$$\frac{\Delta S_M}{k}=(N_1+N_2)\ln N-N_1\ln N_1-N_2\ln(nN_2)$$

$$+(n-1)\left[\frac{z'}{2n}N_1+\left(\frac{z'}{2}-1\right)N_2\right]\ln\left[1-\frac{2nN_2}{z'N}\right]$$

$$-(n-1)\left(\frac{z'}{2}-1\right)N_2\ln\left[1-\frac{2}{z'}\right]. \qquad (29)$$

The partial molal entropies of dilution, divided by the gas constant per mole, are

$$\frac{\Delta\bar{S}_1}{R}=\left(\frac{\partial\left(\frac{\Delta S_M}{k}\right)}{\partial N_1}\right)_{N_2}=-\ln\left(\frac{N_1}{N}\right)+\frac{(n-1)z'}{2n}\ln\left(1-\frac{2nN_2}{z'N}\right) \qquad (30a)$$

$$=-\ln N_1{}^*-\left(1-\frac{1}{n}\right)N_2{}^*g_1 \qquad (30b)$$

and

$$\frac{\Delta\bar{S}_2}{R}=\left(\frac{\partial\left(\frac{\Delta S_M}{k}\right)}{\partial N_2}\right)_{N_1}=-\ln\left(\frac{nN_2}{N}\right)+$$

$$(n-1)\left(\frac{z'}{2}-1\right)\ln\left(1+\frac{2N_1}{(z'-2)N}\right) \qquad (31a)$$

$$=-\ln N_2{}^*-(1-n)N_1{}^*g_2. \qquad (31b)$$

Here $N_1{}^*$ and $N_2{}^*$ are volume fractions and

$$g_1=1+\frac{N_2{}^*}{z'}+\frac{4}{3}\frac{(N_2{}^*)^2}{(z')^2}+\ldots+\frac{1}{i}\left(\frac{2N_2{}^*}{z'}\right)^{i-1}+\ldots \qquad (32)$$

$$g_2 = 1 - \frac{N_1^*}{z'-2} + \frac{4}{3}\frac{(N_1^*)^2}{(z'-2)^2} + \ldots + \frac{(-1)^{i-1}}{i}\left(\frac{2N_1^*}{z'-2}\right)^{i-1} + \ldots \quad (33)$$

The variation of these functions with composition, for various values of z', is shown graphically in FIGURES 2 and 3.
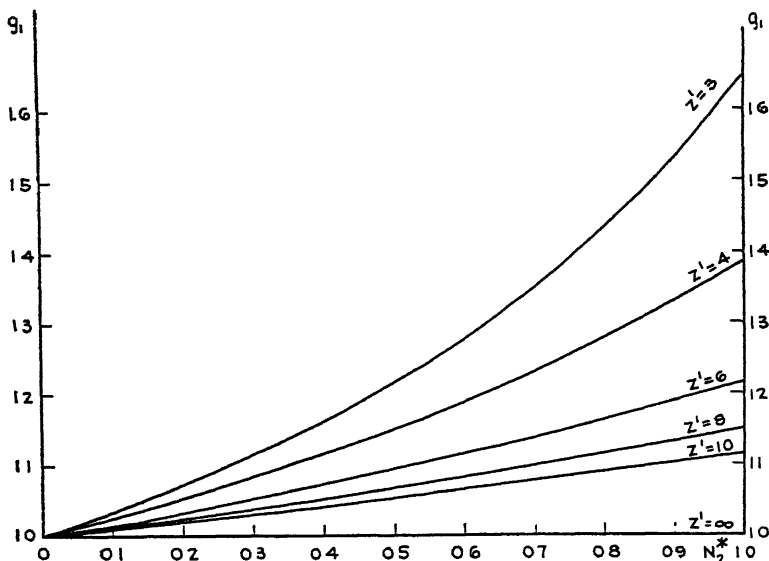

FIGURE 2. Showing the variation of the factor g_1 with the volume fraction N_2^*. See equations (30) and (32).
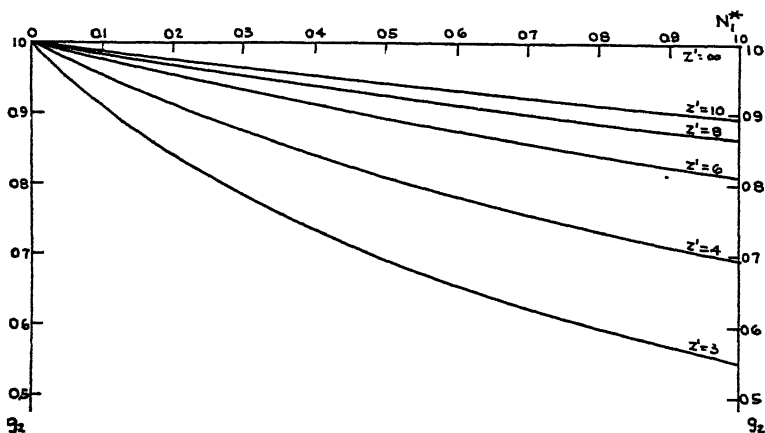
FIGURE 3. Showing the variation of the factor g_2 with volume fraction N_1^*. See equations (31) and (33).

From the thermodynamic relations

$$\ln a_1 = -\frac{\Delta \overline{S}_1}{R} + \frac{\overline{L}_1}{RT} \qquad\qquad 34$$

and

$$\ln a_2 = -\frac{\Delta \overline{S}_2}{R} + \frac{\overline{L}_2}{RT}, \qquad\qquad (35)$$

where $\overline{L}_1$ and $\overline{L}_2$ are the partial molal heats of dilution, we obtain

$$a_1 = N_1{}^{*}\left(1 - \frac{2}{z'}N_2{}^{*}\right)^{-\left(\frac{n-1}{2n}\right)z'} \exp\left(\frac{\overline{L}_1}{RT}\right) \qquad (36a)$$

$$= N_1{}^{*} \exp\left[\left(1 - \frac{1}{n}\right)N_2{}^{*}g_1 + \frac{\overline{L}_1}{RT}\right] \qquad (36b)$$

and

$$a_2 = N_2{}^{*}\left(1 + \frac{2}{z'-2}N_1{}^{*}\right)^{-(n-1)\left(\frac{z'}{2}-1\right)} \exp\left(\frac{\overline{L}_2}{RT}\right) \qquad (37a)$$

$$= N_2{}^{*} \exp\left[(1 - n)N_1{}^{*}g_2 + \frac{\overline{L}_2}{RT}\right] \qquad (37b)$$

These equations satisfy the Gibbs—Duhem—Margules relationship. They reduce to Raoult's law if the heat of mixing is zero and $n = 1$. For $n = 2$ they are in agreement with the results of Fowler and Rushbrooke[10] and Chang[11,12].

For close-packing of molecules and submolecules, z would have the value 12 and (*cf.* equation (27)) z' would not be far different. In any hypothetical solution conforming closely to the assumptions of our statistical calculation (spherical molecules and submolecules, etc.), z (and z') would have a value less than 12, such as 8 or 10. For such values of z and z', g_1 and g_2 both differ but little from unity over the whole range of composition from $N_1{}^{*} = 1$ to $N_2{}^{*} = 1$. In actual solutions of chain molecules we should expect the positioning and orientation of each molecule and submolecule to be considerably limited by the shapes and spatial distribution of its neighbors—as well as by the occupancy or vacancy of neighboring sites. This fact may perhaps be taken care of by the use of smaller values of z', determined empirically. However, it would be unreasonable to expect the effective z' value to be independent of composition, especially when the proportion of chain molecules is large.

THE EFFECT OF A DIFFERENCE IN VOLUME BETWEEN A TYPE 1 MOLECULE AND A TYPE 2 SUBMOLECULE

The effect, on the entropy of mixing and derived quantities, of a difference in volume between a type 1 molecule and a type 2 submolecule, is difficult to determine. A satisfactory treatment of this volume effect, even for spherical solvent and solute molecules, has not yet been published.[14] However, with certain approximations, apparently of a minor nature, we can carry through a statistical treatment of one special case, *i. e.*, that in which each type 2 molecule is composed of n segments, each containing p colinear submolecules, assuming as before that each type 2 submolecule is similar in size and shape to each type 1 molecule (See FIGURE 4)
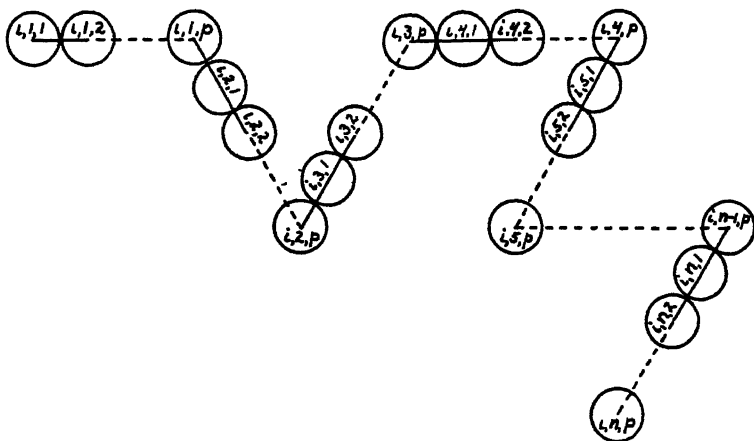
FIGURE 4. Illustrating the designation of submolecules in the i-th type 2 molecule (second model).

We designate a given submolecule by means of three subscripts: i, j and k, denoting the number of the molecule, the number of the segment, and the number of the submolecule in that segment. Making the approximation that the probability that a given site is occupied (when the submolecule i, j, k is about to be placed) is the same for all submolecules in the same segment (*i. e.*, is independent of the value of k), we have, corresponding to equation (14),

$$f_{ij} = f_0 + \frac{np(i-1)(1-f_0)}{N}. \tag{38}$$

[14] See Ref. 12, p. 366.

The probability that a given site is not occupied is $1 - f_{,}$. The probability that a given site is unoccupied and that $p - 1$ other sites, all on a straight line in a given direction, are also unoccupied is $(1 - f_{,})^p$. The probability that at least one of the p closest sites in a given direction from a given site is occupied is

$$f'_{,j} = 1 - (1 - f_{,j})^p. \tag{39}$$

The probability that none of the p closest sites in each of y given directions is occupied is $1 - f'_{,}{}^y_{,j}$. Likewise, for $p - 1$ sites in each of z directions, this probability equals $1 - f''_{,}{}^z_{,j}$, where

$$f''_{,j} = 1 - (1 - f_{,j})^{p-1} \tag{40}$$

The factor corresponding to $\left[1 - \dfrac{2(n-1)f_{,j}}{nz}\right]^{n-1}$ in equation (19) depends on y, z, n, p and $f_{,j}$, in a complicated way. One can show that, if n is large and p is not too small, this factor can be replaced, approximately, by $\left(1 - \dfrac{2f_{,j}}{\gamma z}\right)^{np}$, where γ probably has a value of about 2 for values of p which are neither very large nor very small.

If n is large we can write:

$$(1 - f_{,j}) \, (1 - f''_{,j}) = (1 - f'_{,j}) = (1 - f_{,j})^p. \tag{41}$$

On the basis of the foregoing discussion, we obtain, for this model,

$$\prod \nu_{,j,k} = \prod_{i=1}^{N_2} \frac{Nzy^{n-1}(1 - f_{,j})^{np}}{\left(1 - \dfrac{2f_{,j}}{\gamma z}\right)^{np}}, \tag{42}$$

corresponding to equation (19).

Proceeding as with the simpler model, we deduce

$$\frac{\Delta S_M}{k} = (N_1 + N_2) \ln N - N_1 \ln N_1 - N_2 \ln (npN_2)$$

$$+ \left[\frac{\gamma z}{2}N_1 + \left(\frac{\gamma z}{2} - 1\right) npN_2\right] \ln \left[1 - \frac{2np\,N_2}{\gamma z N}\right] \tag{43}$$

$$- \left(\frac{\gamma z}{2} - 1\right) npN_2 \ln \left(1 - \frac{2}{\gamma z}\right),$$

which is equivalent to equation (29) for large values of n, except for the substitution of np for n and of γz for z'. Equations (30) to (37) also apply to this model, provided these substitutions are made.

Since n in the first model considered and np in the one treated later both denote the ratio of the volume of a type 2 molecule to that of a type 1 molecule, we can write for both models, in place of equations (30), (31), (36) and (37):

$$\frac{\Delta \bar{S}_1}{R} = - \ln N_1{}^* - \left(1 - \frac{V_1}{V_2}\right) g_1 \, N_2{}^* \qquad (44)$$

$$\frac{\Delta \bar{S}_2}{R} = - \ln N_2{}^* - \left(1 - \frac{V_2}{V_1}\right) g_2 \, N_1{}^* \qquad (45)$$

$$a_1 = N_1{}^* \exp\left[\left(1 - \frac{V_1}{V_2}\right) g_1 \, N_2{}^* + \frac{\bar{L}_1}{RT}\right] \qquad (46)$$

$$a_2 = N_2{}^* \exp\left[\left(1 - \frac{V_2}{V_1}\right) g_2 \, N_1{}^* + \frac{\bar{L}_2}{RT}\right] \qquad (47)$$

One is tempted to guess that these equations, with $g_1 = g_2 = 1$ and with V_1 and V_2 replaced by the *partial* molal volumes $\bar{V}_1$ and $\bar{V}_2$, give correctly (provided complete randomness of molecular distribution and orientation exists) the effect on the entropies of dilution and the activities of the relative molecular *sizes*, regardless of their *shapes* and *flexibilities*—these last factors being taken care of by the functions g_1 and g_2. This possibility, however, needs to be explored further.

THE EFFECT OF DIFFERENCES IN FLEXIBILITY OF THE CHAIN MOLECULES

The introduction of rigidity into a fraction $\dfrac{p-1}{p}$ of the bonds in a chain affects the entropies of dilution in only a minor way, by changing the effective value of z. One can conclude from this that these entropies should also be affected only slightly, and in a similar manner, by differences in flexibility of the chain at each junction between submolecules. That this should be the case can also be seen from the fact that this flexibility is represented in equations (19) and (21) by the quantity y, which does not enter at all into equation (29) for the entropy of mixing. In actual solutions we might expect y to vary somewhat with the concentration of the solution, being a minimum usually for the pure liquid of type 2. If so, y should enter into equation (29) and later equations. Neglect of this may introduce considerable error in the region where $N_2{}^*$ is large, but little in the region of low concentration of the chain molecules.

THE HEAT OF MIXING

Elementary statistical considerations[15] lead to the conclusion that the

[15] *Cf.* Porter, A. W.   Trans. Faraday Soc. **16**: 336.   1921;   also Staverman, A. J., & van Santen, J. H.   Rec. trav. chim. **60**: 76.   1941.

partial molal heat of mixing of each component can be represented by a series of terms involving powers of the volume fraction of the other component, the first term being the square term:

$$\overline{L}_1 = \alpha_1(N_2{}^*)^2 + \beta_1(N_2{}^*)^3 + \ldots \ldots \tag{48}$$

$$\overline{L}_2 = \alpha_2(N_1{}^*)^2 + \beta_2(N_1{}^*)^3 + \ldots \ldots \tag{49}$$

Van Laar,[16] Scatchard,[17] and Hildebrand,[18] on the basis of certain assumptions which we shall not take the space to discuss here. have deduced the relations

$$\overline{L}_1 = K_{1,2} \, V_1(N_2{}^*)^2 \tag{50}$$

and

$$\overline{L}_2 = K_{1,2} \, V_2(N_1{}^*)^2, \tag{51}$$

where $K_{1,2}$ is related to the molal energies of vaporization (ΔE) of the pure components, according to the equation

$$K_{1,2} = \left[\left(\frac{\Delta E_1}{V_1}\right)^{\frac{1}{2}} - \left(\frac{\Delta E_2}{V_2}\right)^{\frac{1}{2}}\right]^2. \tag{52}$$

Although equations (50) and (51) are derived only for what Hildebrand calls "regular solutions," in which the entropy of mixing is the same as for ideal solutions, we may write for our more general case, as a first approximation,

$$\overline{L}_1 = \alpha_1(N_2{}^*)^2 \tag{53}$$

for small $N_2{}^*$, and

$$\overline{L}_2 = \alpha_2(N_1{}^*)^2 \tag{54}$$

for small $N_1{}^*$. We should not expect these to hold well for concentrated solutions, especially if the heats of mixing are negative. This does not matter much for dilute solutions of really long-chain compounds, since the contribution of the heat term to the free energy of mixing is small in comparison with the contribution of the entropy term.

Using equation (53) and the first two terms of equation (32), we obtain, on substituting into equations (30b) and (34) and replacing n by $\overline{V}_2/\overline{V}_1$,

$$\ln a_1 = \ln N_1{}^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2{}^* + \mu_1(N_2{}^*)^2 \tag{55}$$

where

[16] **van Laar. J. J.** Z. physik. Chem. **A 137:** 421. 1928.
[17] **Scatchard, G.** Chem. Rev. **8:** 321. 1931.
[18] **Hildebrand, J. H.** J. Am. Chem. Soc., **57:** 866. 1935; Chem. Rev. **18:** 315. 1936; "Solubility of Non-Electrolytes," Second Edition, Reinhold Publishing Corporation, New York. p. 73. 1936.

$$\mu_1 = \frac{1 - \dfrac{1}{n}}{z'} + \frac{\alpha_1}{RT} . \tag{56}$$

A corresponding expression for $\ln a_2$ can be similarly deduced.*

COMPARISON WITH EXPERIMENT

Equation (55) is readily susceptible to experimental test. The single constant, μ_1, remains to be determined empirically. Experimental activity values can, of course, be derived from data of various sorts. For example, the activity of the solvent equals the ratio of the partial pressure of the solvent over the solution to the vapor pressure of the pure liquid solvent,

$$a_1 = p_1 \ p_1^{\circ}, \tag{57}$$

provided that the vapor behaves as a perfect gas. The changes in the freezing point and the boiling point from the values for the pure solvent are related to the activity of the solvent by the equations

$$\vartheta_f = -\frac{RT_f^2}{\Delta H_f} \ln a_1 \tag{58}$$

and

$$\vartheta_b = \frac{RT_b^2}{\Delta H_v} \ln a_1, \tag{59}$$

where ΔH_f and ΔH_v are the heats of fusion and vaporization and T_f and T_b are the absolute temperatures of the freezing and boiling points of the pure solvent. The osmotic pressure is likewise related to the activity of the solvent by the equation

$$\Pi = -\frac{RT}{\overline{V}_1} \ln a_1 . \tag{60}$$

The best data for our purpose are probably those which have been collected by Meyer and his coworkers in Geneva. Typical data of theirs and some from other sources are shown graphically in FIGURES 5 to 16[19].

---

* The constants μ_1 and μ_2 are related by the equation $\overline{V}_2\mu_1 = \overline{V}_1\mu_2$.

[19] At the Atlantic City American Chemical Society Meeting, various graphs were shown in which $\ln a_1 - \ln N_1^*$ was plotted against N_2^*. Since the theory predicts a rectilinear relation, when so plotted, provided μ_1 is zero, this method shows well the general agreement between theory and experiment, as well as the magnitude and relative unimportance at small N_2^* values of the $\mu_1 (N_2^*)^2$ term. On the other hand, minor deviations at small N_2^* values do not show up well, also the theoretical and actual large deviations from Raoult's law are not plainly indicated. For these reasons the experimental data and theoretical curves are represented here in a different manner, as detailed above.
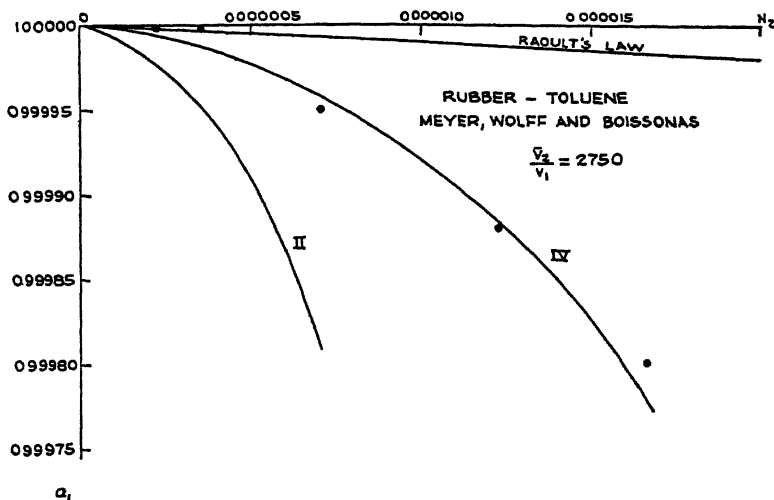
FIGURE 5.

FIGURES 5 to 16. Activity versus mole fraction for certain binary solutions. The straight line represents Raoult's law ($a_1 = N_1$). The equations for the other curves are:

$$\text{I. } a_1 = \exp\left[\ln N_1 + \frac{\alpha_1}{RT}(N_2^*)^2\right]$$

$$\text{II. } a_1 = \exp\left[\ln N_1^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2^*\right]$$

$$\text{III. } a_1 = \exp\left[\ln N_1^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2^* + \frac{\alpha_1}{RT}(N_2^*)^2\right]$$

$$\text{IV. } a_1 = \exp\left[\ln N_1^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2^* + \mu_1(N_2^*)^3\right]$$

The systems are listed, with the pertinent constants, in TABLE 1.

The straight lines in these figures represent Raoult's law. For purposes of comparison, the scales were so chosen as to make the slopes of these lines uniformly 45°, except in two cases (FIGURES 5 and 6), for which such a choice would make the experimental activities lie practically on the axis of ordinates. Curve I, in each case, represents the activity of the solvent if the solution were regular, assuming equation (53) and using a value of α estimated from the experimental data. Curves II and III represent activities calculated with the aid of equation (55), setting $\mu_1 = 0$ and $\mu_1 = \alpha_1 RT$, respectively. Curves IV are similarly obtained, except that an empirical value of μ_1, derived from graphs of

$$\ln a_1 - \left[\ln N_1^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2^*\right] \text{ vs. } (N_2^*)^2 \text{ (FIGURES 17 to 19), is used in}$$

each case.

The constants for these curves are listed in TABLE 1.

TABLE 1

Figure No.	System	$\bar{V}_2/\bar{V}_1$	μ₁	α_1/RT	Temperature (°C.)	Method	Reference
5	Rubber—toluene	2750	0.41		24.4	Osmotic pressure	6
6	Rubber—toluene	2750	.41		25–30	Vapor pressure	6
7	Nitrocellulose—cyclohexanone	450	.15		25	Osmotic pressure	4
8	Cellulose triacetate—tetrachloroethane	300	–3.0		24.4	Osmotic pressure	8
9	Dioleyl thapsate—chloroform	10.9	–0.12	–0.9	50	Vapor pressure	2
10	Dioleyl thapsate—carbon tetrachloride	9.0	+ .15	0.0	50	V. p. and b.pt.	2
11	Oleyl oleate—carbon tetrachloride	6.4	+ .10	– .3	50	V. p. and b.pt.	2
12	Oleyl oleate—cyclohexane	5.7	+ .29	+1.0	50	Vapor pressure	2
13	Oleyl oleate—n-hexane	4.7	+ .35	+0.55	50	Vapor pressure	2
14	Cetyl palmitate—cyclohexane	5.1	– .55		82	Boiling point	2, 20
15	Butyl sebacate—benzene	3.8	0.00		18	Vapor pressure	3, 21
16	Butyl valerate—benzene	2.1	–0.04		18	Vapor pressure	3, 21

20 Eijkman, J. F.   Chem. Weekblad. 1: 47.   1903.
21 Bröñsted, J. N., & Colmant, P.   Z. physik. Chem. A 168: 381.   1934.

FIGURE 6

FIGURE 7.

CELLULOSE TRIACETATE -
TETRACHLOROETHANE

$$\frac{V_2}{V_1} = 300$$

II

HAGGER AND VAN DER WYK

IV
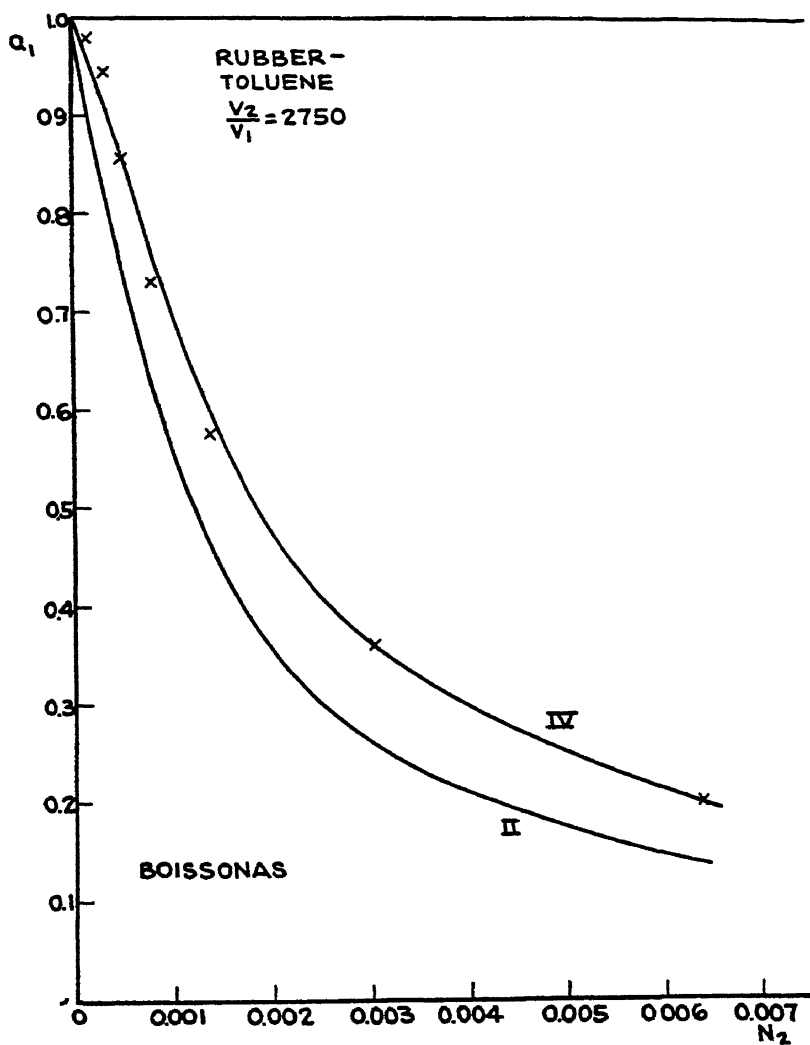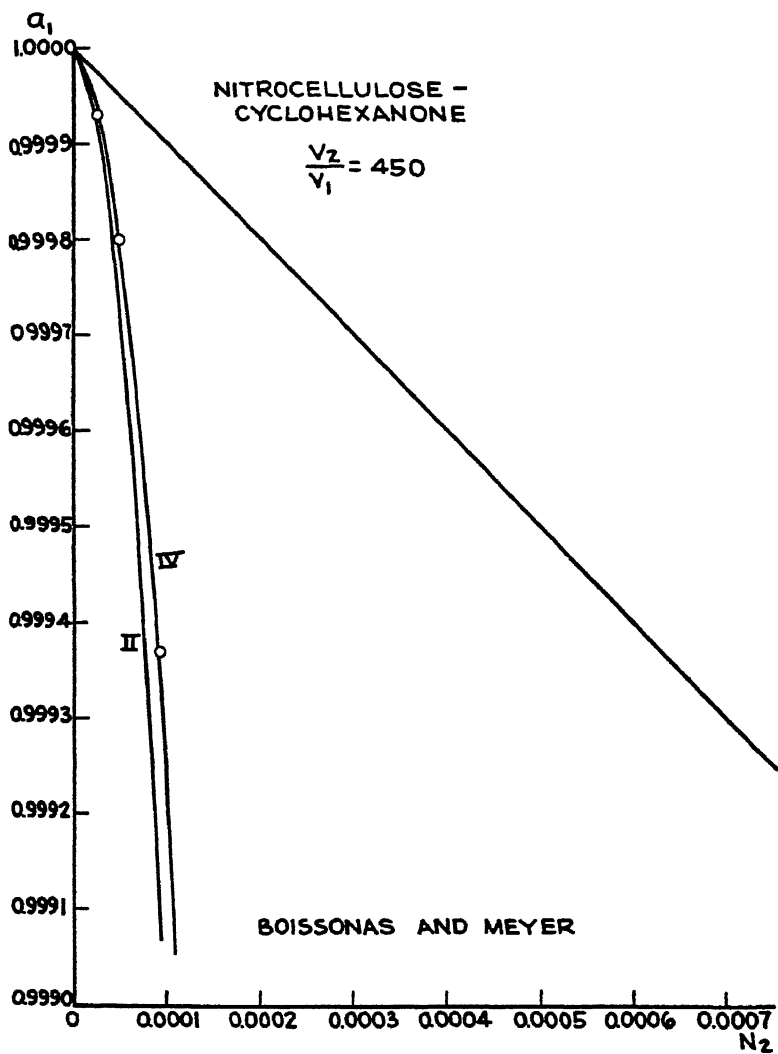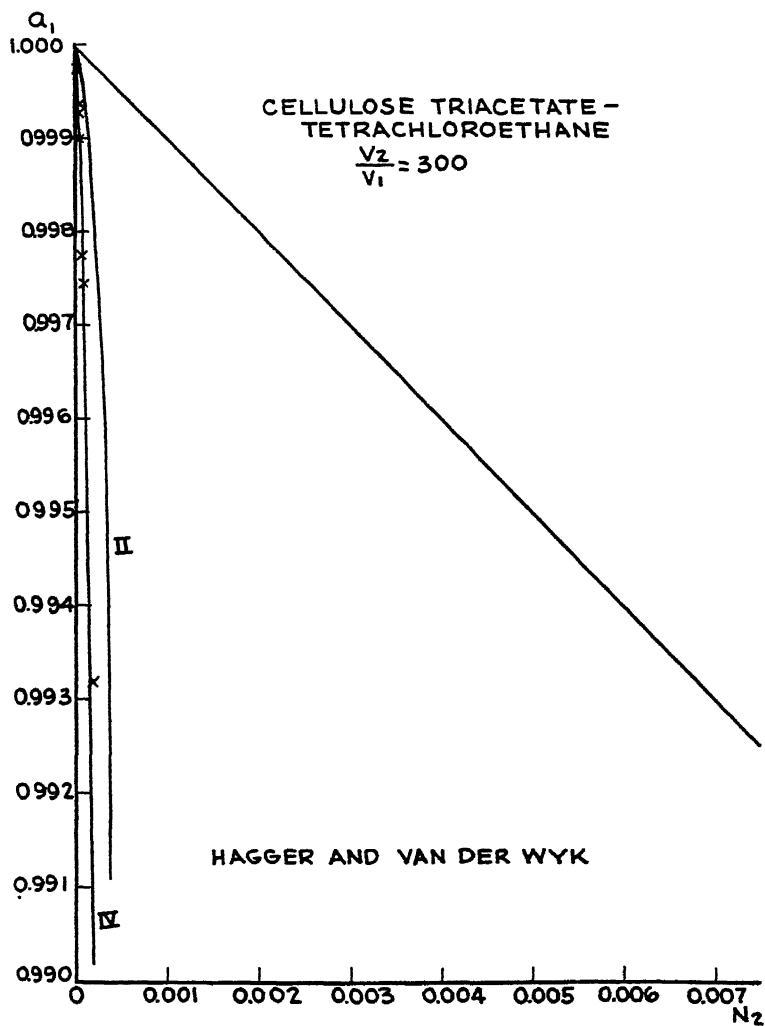
FIGURE 8.

The molal volume ratio, $\bar{V}_2 \; \bar{V}_1$, has been computed from estimated values for the pure liquids. In some instances these are doubtless inaccurate, but this inaccuracy matters little for our present purpose, since the volume ratio occurs only in a small, relatively unimportant term. The value of μ_1 obtained empirically, however, is considerably influenced by an error in the volume ratio.
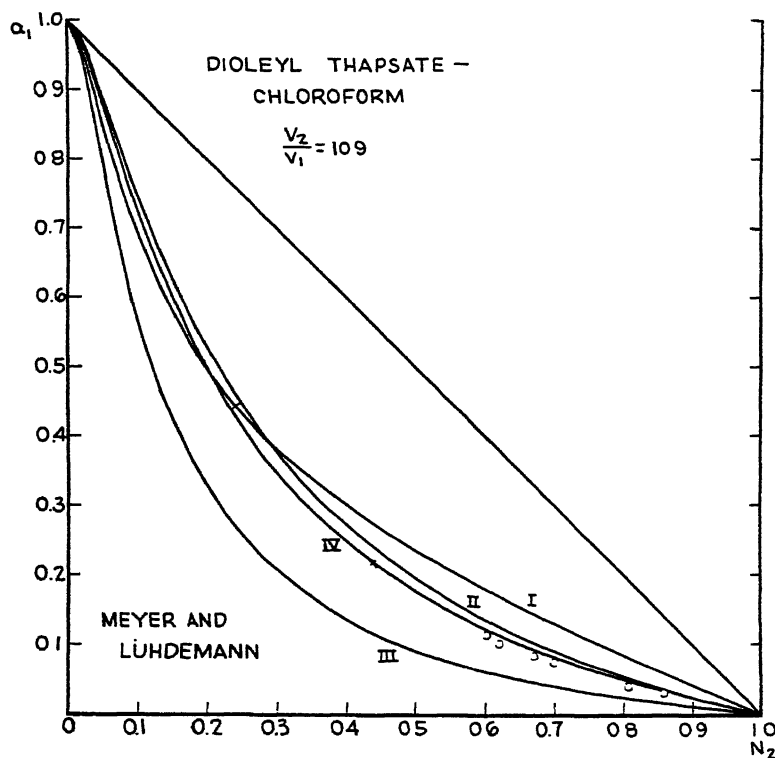
FIGURE 9.

It is evident from the figures that the agreement with equation (55) is quite good, whether the volume ratio is small or very large. The empirical constant, μ_1, which must be used in most cases to obtain close agreement when N_2^* is not small, approximately takes care of the heat of mixing effect, the difference between the entropy of mixing for infinite coordination number and that for a small effective average coordination number, and any deviations from the "complete" randomness assumed in the theoretical treatment. These deviations from complete

randomness must be quite large when the heat of mixing positive or negative) is large. As more data become available, we can hope to study these factors more quantitatively.*

Deviations from complete randomness must also be large, at room temperature or lower, in pure liquid paraffins and such of their derivatives as consist primarily of $(-CH_2-)_n$ chains, and also in their concen-

DIOLEYL THAPSATE –
CARBON TETRACHLORIDE

$$\frac{V_2}{V_1} = 9.0$$

MEYER AND LÜHDEMANN
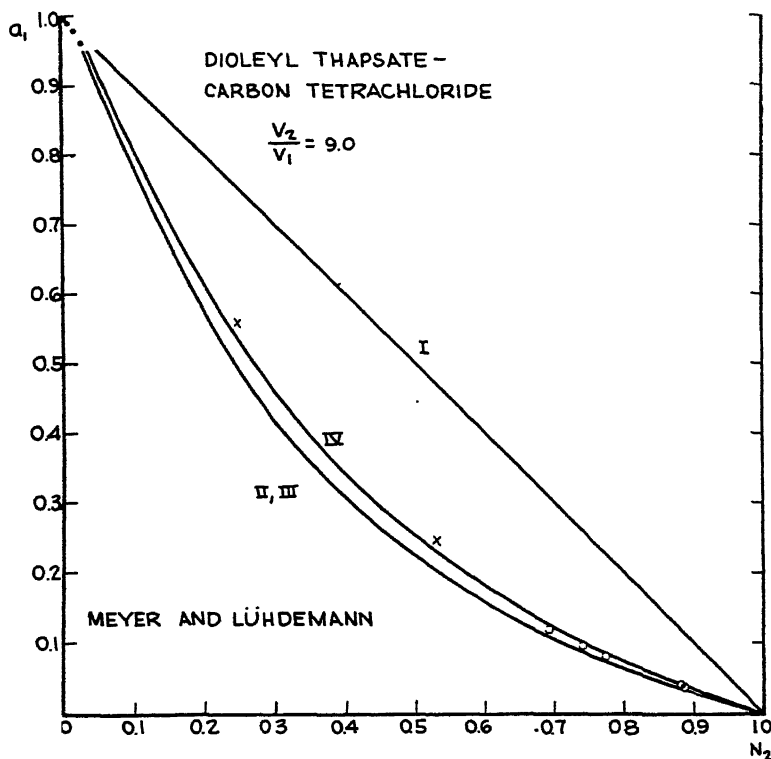
FIGURE 10.

trated solutions—especially solutions with other paraffins—as a result of the known tendency of such molecules to align themselves with their chain axes more or less straight and parallel to each other, within small regions. With most of the molecules so oriented, one would expect from theoretical considerations, as Hildebrand[22] has shown, that Raoult's

---

* Professor Scatchard has kindly pointed out that the differences between α_1 'RT and the experimental values of μ_1 may be due in part to volume changes on mixing. *Cf* Scatchard, G. Trans. Faraday Soc. **33**: 160. 1937.

[22] Hildebrand, J. H. Jour. Amer. Chem. Soc. **59**: 794. 1937; Jour. Phys Chem. **43**: 109. 1939.

law would be closely approximated, provided the heat of mixing is negligible. This expectation has been verified experimentally by Hildebrand and Sweny[23] for solutions of hexadecane and hexane at 25° C. On the contrary, Meyer[5] has reported that (unpublished) data obtained by Boissonas on solutions of octadecane and hexane show large

OLEYL OLEATE –
CARBON TETRACHLORIDE

$\dfrac{\bar{V}_2}{\bar{V}_1} = 6.4$

MEYER AND LÜHDEMANN
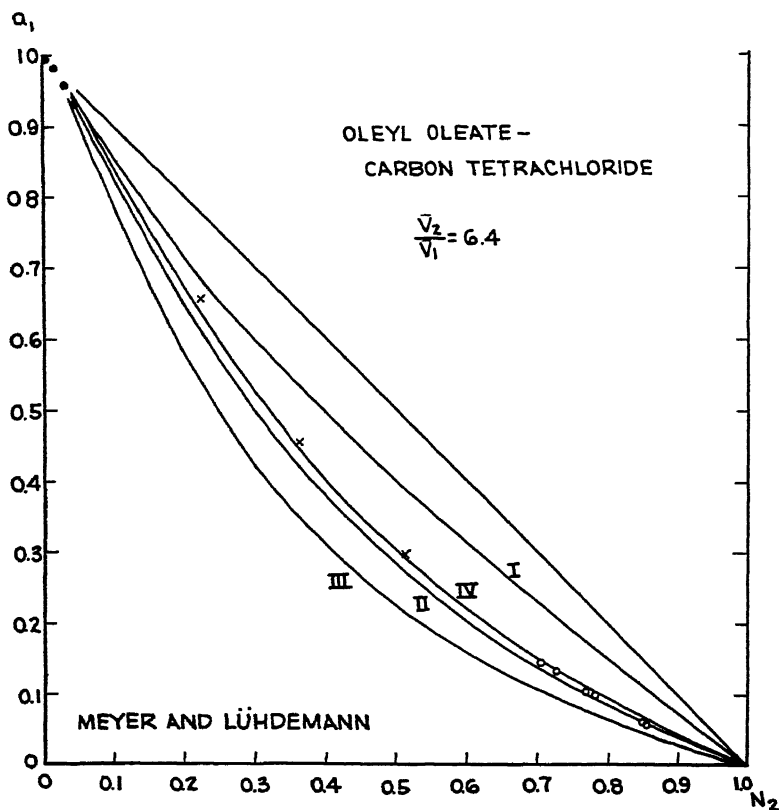
FIGURE 11.

deviations between the experimental entropies of mixing and the ideal values. Perhaps this system was studied at a higher temperature. The higher the temperature the less alignment of the chains would be expected. On the other hand, Hildebrand and Sweny's data agree with equation (55) just as well as with Raoult's law, if we set $\mu_1 = 0.20$ (FIGURE 20).

23 Hildebrand, J. H., & Sweny, J. W.   Jour. Phys. Chem. **43**: 297.   1939.

OLEYL OLEATE –
CYCLO HEXANE

$\dfrac{\bar{V}_Z}{\bar{V}_1} = 5.7$

I

III

IV

II

MEYER AND LÜHDEMANN
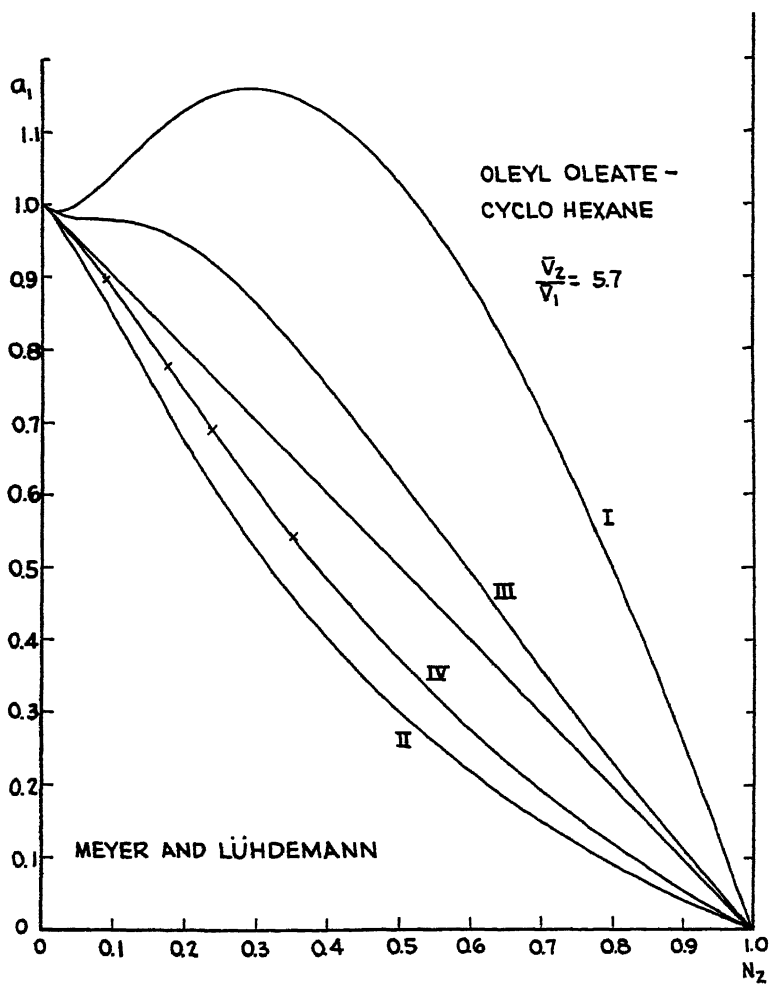
FIGURE 12.

DISCUSSION

In view of the agreement obtained with experiment, we can proceed with confidence to apply our theoretical equations to various thermodynamic properties of solutions. If the solutions of interest are dilute (N_2^* small), we can write

$$\ln N_1^* = -N_2' - \frac{N_2'^2}{2} - \frac{(N_2^*)^3}{3} - \ldots \ldots \tag{61a}$$

$$\approx -N_2^* - \frac{(N_2')^2}{2} \tag{61b}$$

OLEYL OLEATE –
N-HEXANE

$\frac{V_2}{V_1} = 4.7$

MEYER AND LÜHDEMANN
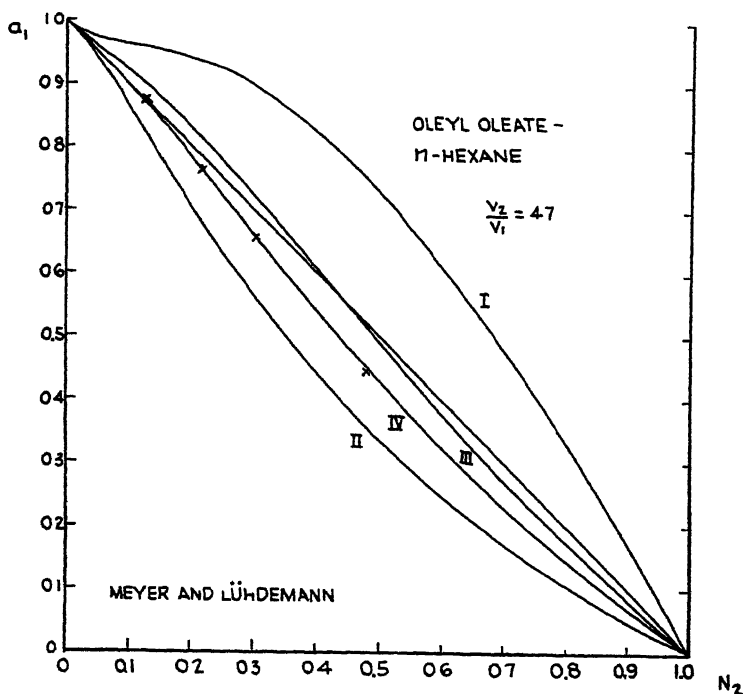
FIGURE 18.

and so, substituting in equation (55),

$$\ln a_1 = -\frac{\overline{V}_1}{\overline{V}_2} N_2^* + \left(\mu_1 - \frac{1}{2}\right)(N_2^*)^2. \tag{62}$$

Inserting this result into equation (60), we deduce

$$\frac{\Pi}{N_2} = \frac{RT}{\overline{V}_2} + \frac{RT}{\overline{V}_1}\left(\frac{1}{2} - \mu_1\right)N_2.$$ (63a)

$$= \frac{RTd_2}{M_2} + \frac{RTd_1}{M_1}\left(\frac{1}{2} - \mu_1\right)N_2,$$ (63b)

in which d and M represent density and molecular weight, respectively. As the volume fraction, N_2, and the weight fraction, W_2, are approximately related, at low concentrations, by the equation

$$N_2 = \frac{W_2 d_1}{d_2},$$ (64)
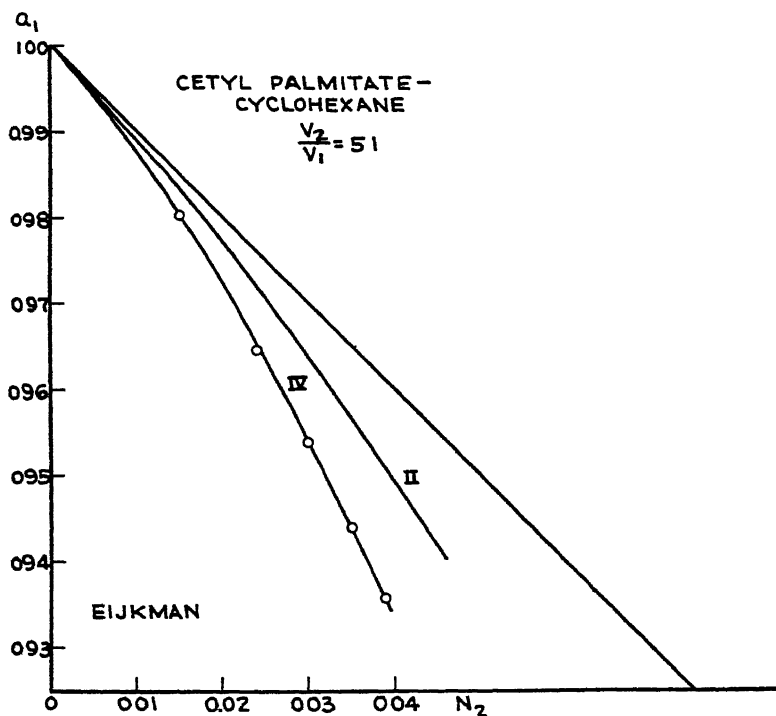
FIGURE 14.

we may write

$$\frac{\Pi}{W_2} = \frac{RTd_1}{M_2} + \frac{RTd_1^3\left(\frac{1}{2} - \mu_1\right)W_2}{d_2^2}.$$ (65)

Hence, if Π W_2 is plotted against W_2, the experimental points should fall on a straight line, provided the solution is sufficiently dilute. For a given solvent, the intercept of this straight line with the axis of ordinates ($W_2 = 0$) should be inversely proportional to the molecular weight of the solute; its slope should depend on both solvent and solute but should be practically the same for solutes which are chain molecules
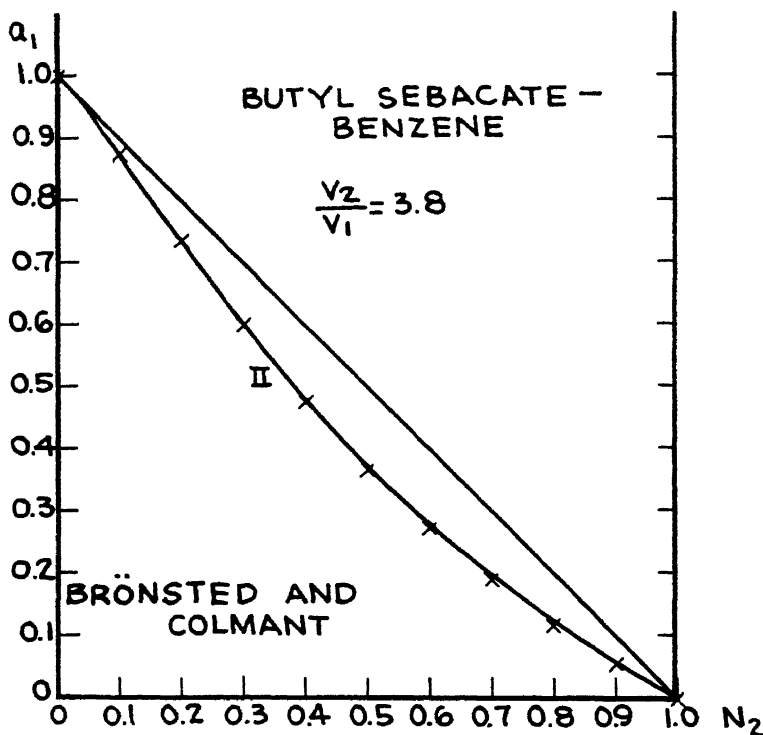
FIGURE 15.

differing in length but otherwise similar; *e. g.*, for polymers of different chain length. These relationships have been known empirically for some time.[24]

Corresponding relations can, of course, be similarly derived, using equations (58) and (59), for the freezing point depression and the boiling point elevation.

[24] Mark, H. "Physical Chemistry of High Polymeric Systems," Interscience Publishers, New York. p. 240. 1940.

BUTYL VALERATE—
BENZENE

$$\frac{\bar{V}_2}{\bar{V}_1} = 21$$

BRÖNSTED AND COLMANT

FIGURE 16.

CELLULOSE TRIACETATE —
TETRACHLOROETHANE

$\mu_1 = -3\,0$

HAGGER AND
VAN DER WYK
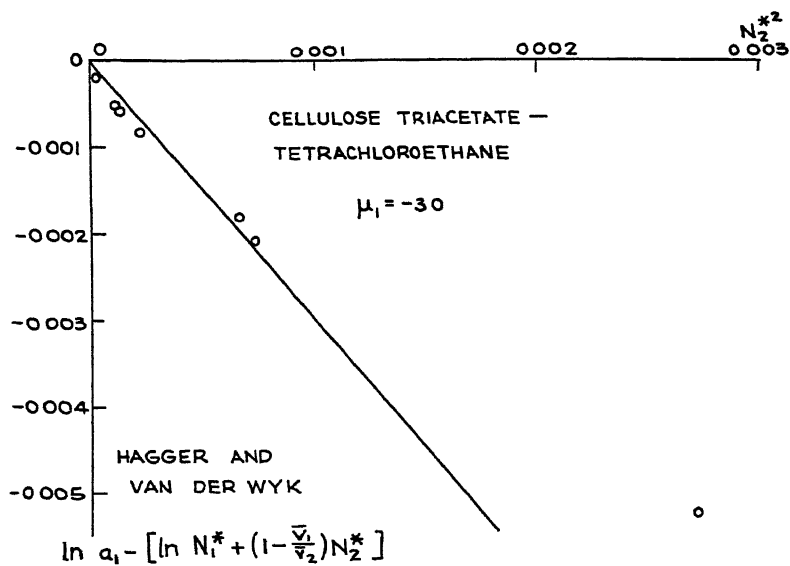
$$\ln\,a_1 - \left[\ln\,N_1^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2^*\right]$$

FIGURE 17.

FIGURES 17 to 19.　Graphs illustrating the empirical determination of μ_1.

CETYL PALMITATE.—
CYCLOHEXANE

$\mu_1 = -0\,55$

EIJKMAN

$$\ln\,a_1 - \left[\ln\,N_1^* + \left(1 - \frac{\overline{V}_1}{\overline{V}_2}\right)N_2^*\right]$$

FIGURE 18.

$$\ln a_1 - [\ln N_1^* + (1 - \tfrac{\overline{v}_1}{\overline{v}_2}) N_2^*]$$

OLEYL OLEATE —
CYCLOHEXANE

$\mu_1 = 0.29$

MEYER AND LÜHDEMANN

N_2^{*2}

FIGURE 19.

$a_1 - N_1$

HEXADECANE – HEXANE

HILDEBRAND AND SWENY

N_2
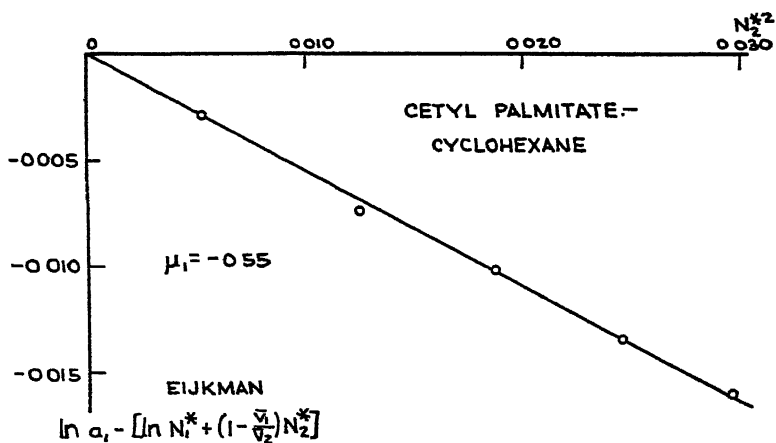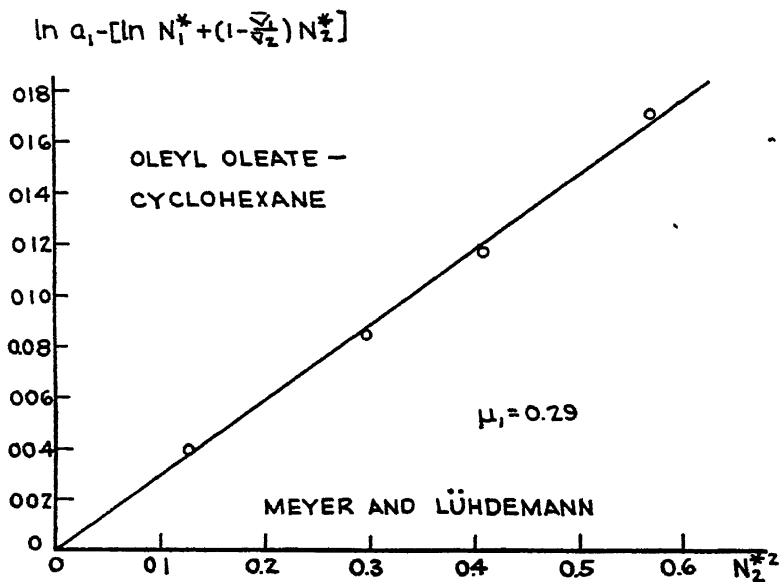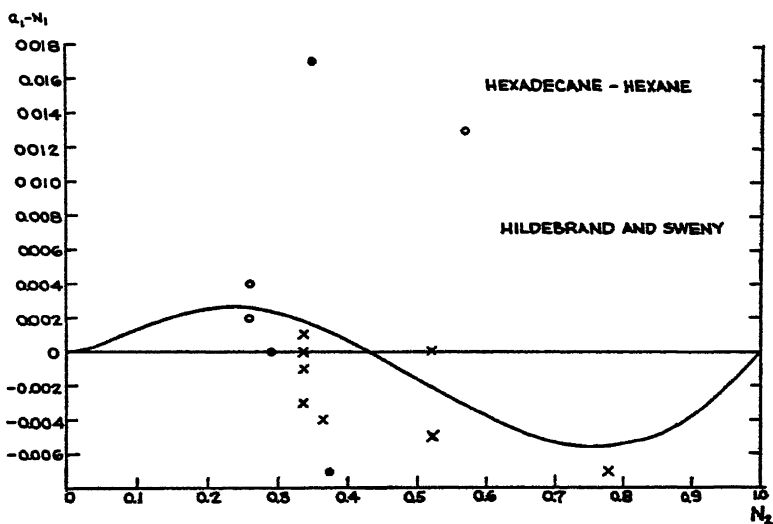
FIGURE 20. Deviations from Raoult's law, for *n*-hexadecane—*n*-hexane solutions at 25°. Data by Hildebrand and Sweny.[23] The curve is for $a_1 = \exp\left[\ln N_1^* + \left(1 - \dfrac{\overline{V}_1}{\overline{V}_2}\right)N_2^* + 0.20\,(N_2^*)^2\right]$.

CONCLUSIONS

On the basis of the new expressions for the activities derived in this paper, a sound theoretical treatment is now possible for the solubility of a long-chain compound in a liquid of low molecular weight and of such a liquid in a gel composed of long chains or of a flexible network (which is practically equivalent, as regards both entropy and heat terms, to a chain of very great length).   A number of interesting conclusions result very simply from such a treatment.   This important subject, however, warrants more detailed consideration than can be given here, hence it will be postponed until a later date.

In conclusion, the writer is pleased to express his thanks for helpful criticism of the manuscript to Dr. S. E. Sheppard of the Kodak Research Laboratories and Professors J. H. Hildebrand, G. Scatchard and J. G. Kirkwood of the University of California, the Massachusetts Institute of Technology, and Cornell University, respectively.

IMMUNOCHEMISTRY*

By

William C. Boyd, Enrique E. Ecker, Michael Heidelberger, San-
ford B. Hooker, Forrest E. Kendall, Stuart Mudd, L. Pillemer,
Joseph E. Smadel, Theodore Shedlovsky, and Charles A. Zittle

CONTENTS

INTRODUCTION TO THE CONFERENCE ON IMMUNOCHEMISTRY

By Michael Heidelberger

From the College of Physicians and Surgeons, New York

The Conference on Immunochemistry which I have the privilege and pleasure of now calling to order is the first under the auspices of the Section on Chemistry and Physics of the New York Academy of Sciences to venture so far afield from the sheltered paths of classical physics and chemistry. This excursion is not entirely inappropriate, for if one examines into the matter more closely it becomes evident that Immunochemistry has made its great advances in recent years with the same organic chemical, physical chemical, and analytical chemical methods that have provided the substantial background and material for the other conferences which have been held. While we in Immunochemistry cannot always reason as rigorously as those in the more formal branches of physics and chemistry we are rapidly progressing in this direction and fundamental principles are becoming more clear. The variety and scope of the papers offered guarantee that this forward movement is being continued.

ANTIGENS OF VACCINIA

By Joseph E. Smadel and Theodore Shedlovsky

From the Laboratories and the Hospital of The Rockefeller Institute for Medical Research, New York

The virus of vaccinia is known to have a complex chemical structure. Protein, nucleoprotein, phospholipid, neutral fat, and carbohydrate have been indentified in preparations of purified elementary bodies of vaccinia in amounts comparable, in a general way, to those of bacteria and mammalian cells.[1] The complexity of the virus is also indicated by observations of another sort; for example, a number of different immune bodies are found in the sera of animals following infection with this agent. These antibodies include neutralizing substances,[2] antibodies against a heat-labile (L) and a heat-stable (S) soluble substance,[3] an agglutinin designated "X",[4] and finally, an antibody against a nucleoprotein which is present in elementary bodies.[5]

The nature of the substance or substances in elementary bodies responsible for inducing immunity and the development of neutralizing

antibodies in animals is not known. We shall review briefly the immunological properties of the other antigens of vaccinia and shall discuss at length the relationship that exists between the heat-labile and heat-stable soluble substances. A summary of the work which led to the recognition of the nucleoprotein extracted from elementary bodies as a new antigen of vaccinia' will serve as an adequate review of the previously recognized antigens

About half the material in a dried, purified preparation of elementary bodies of vaccinia is brought into solution by treatment with N 20 NaOH at 56° C. for 15 minutes. The undissolved portion consists of non-infectious ghosts of elementary bodies which have a lower density by ultracentrifugation studies, which stain less deeply by the silver technique, and which appear less brilliant by dark-field illumination than do active virus particles. These particles can be separated from the soluble extracted material by ultracentrifugation at 30,000 r.p.m. A non-infectious solution obtained in this manner by alkaline extraction of virus is rich in a nucleoprotein that contains 14.5 per cent nitrogen and 6 per cent nucleic acid of the thymus type. Electrophoresis studies show that the nucleoprotein constitutes about 90 per cent of the material in the extracts (FIGURE 1) and that this major constituent has a mobility

FIGURE 1. Electrophoretic pattern of alkaline extract.

value of 6.4×10^{-5} cm. sec. per volt cm. in 0.05μ (ionic strength) veronal buffer solution at pH 8.75. The nucleoprotein is soluble at pH values above 8.0 and is partially or completely insoluble in the pH range between 4.5 and 7.5. Serological studies on the nucleoprotein have been carried out in solutions buffered at pH 8.6.

Precipitins which react with this nucleoprotein of vaccinia have been demonstrated in sera of members of several species of animals following hyperimmunization with active virus. Furthermore, rabbits repeatedly injected with inactivated virus particles or with non-infectious alkaline extracts of virus also develop precipitins for the nucleoprotein antigen (NP). Absorption experiments of the type summarized in TABLE 1 clearly indicate that NP-antibodies differ from L-, S-, and X-antibodies of vaccinia. From the results presented in the table it is apparent that hyperimmune rabbit serum precipitated with solutions containing LS-,

TABLE 1

DEMONSTRATION OF MULTIPLE ANTIBODIES IN VACCINAL ANTISERUM

Hyperimmune rabbit serum Absorbed with	Test antigen	Precipitin reactions with solutions of antigen — Dilution of test antigen						Agglutination reactions with suspensions of virus — Dilution of serum									
		1:4	1:8	1:16	1:32	1:64	1:128	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048	1:4096
Unabsorbed	LS	±	+++	++±	+++	+++	±	±	±	±	±	±	±	±	−	−
	S	+++	+++	++±	+++	+++	+	+++	+++	+++	++	+	±	±		
	NP	+++	−	++±	++±	++	±	+	−	−	−	−	−			
S-antigen	LS	±	±±±	±±±	++±	+++	±±	++	++±	+++	+++	±	±	±	−	−
	S	+	±±±	+++	+±	+±	±	++	++±	+++	+++	±				
	NP	±	+	+	±±	++	++	++	++±	+++	+++	±	±	−		
LS-antigen	LS	±±	±±±	±±±	±	−	±	++	+++	+++	++	±				
	S	+	+±±	+++	±+	±	+	++	+++	+++	++	−				
	NP	±	±	±	−	±	−	++	±	±	±	−	−			
NP-antigen	LS	±	±±±	±±±	±	±	±	++	+++	+++	++	±				
	S	+	±±±	±±±	±	−	−	++	+++	+++	++	−	−			
	NP	±	−	±	−	−	−	−	±	−	±					
LS- and NP-antigens	LS				−	−	−	++	+++	++±	++	±	±			
	S	−		−	−	−	−	−	+++	+++	++	++	−	−		
	NP			−	−	−	−	±	+++	+++	+	+				

A 1 8 dilution of serum no. 1601 used throughout precipitin tests.

B lot no 1005 diluted 1 6 used throughout agglutination tests.

S-, and NP-antigens and that it agglutinated suspensions of elementary bodies. Absorption with S-antigen removed S-precipitins but left undiminished the L- and NP-antibodies. Similarly, absorption with LS-antigen resulted in a loss of L- and S-antibodies but did not affect the NP-antibodies; the agglutinating titer of this absorbed serum was reduced. In a like manner, the hyperimmune serum after treatment with NP-antigen no longer precipitated with this substance but still contained L- and S-antibodies and still agglutinated the virus. Finally, when removal of all demonstrable precipitins was accomplished by absorption with LS and NP there still remained some agglutinins for the virus particles. Since each of the precipitating antibodies is capable of aggregating elementary bodies the residual agglutinating substance left after removing the precipitins may be designated X-agglutinin. The neutralizing power of the unabsorbed hyperimmune serum, of the portion absorbed free of NP-antibodies, and of the portion absorbed free of all precipitins was essentially the same. Thus it appears that neutralizing antibody is distinct from NP-precipitin; earlier work[6] had indicated that L- and S-antibodies have little or no neutralizing capacity. Further evidence for considering that the nucleoprotein, in its present form at least, is not the substance responsible for the production of immunity, or of neutralizing antibody may be drawn from the experiment in which normal rabbits were injected with solutions of nucleoprotein. All of the animals failed to develop either immunity or appreciable amounts of neutralizing substances, although they did develop precipitins for the antigen and some agglutinins for the virus.

A problem which has intrigued workers in the field of vaccinia is the relationship which exists between the two non-infectious soluble antigens of vaccinia, L and S. That these two antigens are closely associated immunologically was recognized by Craigie and Wishart[3] several years ago. They observed that the absorption of dermal filtrate, which is rich in L- and S-antigens, with either L- or S-antibody removed completely both of the serologically active substances. Because of these findings, Craigie and Wishart[3] stated that "the L and S antigens are different antigenic components of a complex L-S antigen rather than two independent antigens." Under certain conditions, however, results of a different type have been obtained in absorption experiments; for example, removal of all demonstrable S-antigen without complete loss of L-antigen has been effected by treating dermal filtrate with S-antibody (Craigie and Wishart,[4] Parker[7]). It should be emphasized that this latter observation is an exception to the rule, and that such results are not regularly reproducible. To account for this fact, Craigie[4] assumed that under

certain conditions the L-S complex might be dissociated into separate L- and S-fractions.

More recently, Smadel and Rivers[8] observed that heated dermal filtrate was capable of specifically inhibiting L-antibody. (Gentle heating destroys the specific precipitability of L-antigen, but does not affect the serological activity of S-antigen.) Subsequently, it was found that other procedures, which resulted in the inactivation of L-antigen without apparent loss of S-antigen, likewise gave preparations which specifically inhibited the L-antibody. Moreover, the protein-like S-antigen was carefully purified by the technique of Parker and Rivers[9] and even this material was shown to inhibit L-antibody. At this time it was also found that purified S-antigen which had been heated in the presence of dilute alkali lost its ability to precipitate with S-antibody as well as its power to inhibit L-antibody, but that this degraded product was still capable of inhibiting S-antibody. On the basis of such serological evidence these authors[8] suggested that L- and S-antigens probably always occurred together in nature in the form of a single substance with two serologically active parts each of which could be degraded independently of the other. For the experiments of others[4, 7] which had been interpreted as indicating dissociation of the complex into free L and S they offered a different explanation. This was based on the presence in certain dermal filtrates of a hypothetical degraded form of the complex antigen which had a native L-portion and a degraded S-portion[5].

Studies on the relationship of L- and S-antigens were hampered by the fact that neither L- nor LS-complex had been isolated in pure form. Comparatively pure preparations of S-antigen had been obtained but it remained to be proved conclusively that the inhibitory power of this protein for L-antibody was dependent upon the presence of a degraded L-portion of LS-complex, or that free L-antigen, in a partially denatured state, was present as a contaminant[3]. A method for concentrating and partially purifying both L- and S-antigens from dermal filtrate has been reported by Craigie and Wishart[3] who found that these antigens could be precipitated at pH 4.5 and redissolved at pH 6.5. This method, with only minor modifications, has been successfully employed by us. The principal change we have made is to concentrate the filtrate ten to twenty times by evaporation through cellophane sausage casing before precipitating the antigens by dialysis against buffer solution at pH 4.5.

Electrophoretic studies in which the moving boundary apparatus of Tiselius was employed were made on whole concentrated dermal filtrates and serological titrations were carried out on the filtrates and on various components which were isolated from them by electrophoretic separa-

tion.[10] It was found that the whole dermal filtrate contained four electrophoretically distinct components which are indicated by the "peaks" labelled I, II, III, and IV in FIGURE 2. The remaining peak, ε, which appears in all the electrophoretic patterns, is due to a concentration change of buffer salts and not to a component in the preparations. At a pH of 7.9 in a buffer of ionic strength 0.05, the fastest moving com-

FIGURE 2. Electrophoretic pattern of concentrated dermal filtrate having L and S titers of 1:1600.

ponent, I, and the slowest moving component, IV, were present in the solution in the largest and smallest amounts, respectively. The other two components, II and III, generally appeared in about equal concentrations in dermal filtrate. On heating the preparation at 70° C. for one-half hour (which destroys the serological precipitability of L-antigen) the slower of these two middle components disappeared from the electrophoretic patterns and the concentration of the faster of these two components was increased as is shown in FIGURE 3. Components I and IV
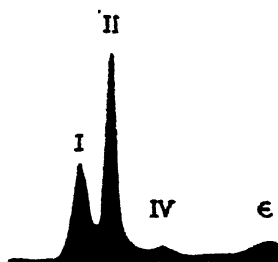
FIGURE 3. Electrophoretic pattern of dermal filtrate after heating.

were each isolated electrophoretically from concentrated filtrate and neither of these was found to precipitate in the presence of anti-vaccinal serum. The serologically active portion of the dermal filtrate was obviously associated with one or both of the middle components. Attempts to separate these components by electrophoretic means were not completely successful due to the fact that the electrical mobilities of the substances were not sufficiently different for adequate resolution. At this point in the investigation we were greatly impressed with the fact

that heating destroyed the precipitability of L and caused a disappearance of component III with an augmentation of component II.   Therefore, the possibility was seriously considered that component III corresponded to L and component II corresponded to S and, furthermore, that heating transformed L to S.   These ideas were subsequently abandoned.

Fractionation of dermal filtrate by precipitation at different values of pH was next undertaken.[10]   Components I and IV remained in solution at pH 4.5.   The material from dermal filtrate which was insoluble at pH 4.5 was only partially soluble at pH 6.5.   Most of the residue could, however, be brought into solution by raising the pH to 8.6.   That portion of the filtrate which was insoluble at pH 4.5 but soluble at pH 6.5 was shown to contain a single component on electrophoresis; the mobility of this substance corresponded to that of component III in whole dermal filtrate.   The fraction of dermal filtrate insoluble at pH 4.5 and also 6.5 but soluble at pH 8.6 was found to consist for the most part of material with a mobility corresponding to that of component II.   Reproductions of electrophoretic patterns obtained with the various fractions isolated from concentrated dermal filtrate (FIGURE 2) at different values of pH are illustrated in FIGURE 4; the results of precipitin titrations with the different solutions are also summarized in this figure.

The solution containing components I and IV was serologically inert when tested with anti-vaccinal sera.   Component II, which was obtained free of component III by repeated fractionation, likewise failed to react with L- and S-antibodies.   The electrically homogeneous fraction III was found to contain practically all of the L- and S-serological activity of the original dermal filtrate (FIGURE 4).   A number of preparations of component III have been studied under a variety of conditions by means of electrophoresis and ultracentrifugation and in each instance the material behaved as a homogeneous substance.   Since component III appeared to be a single molcular substance containing L- and S-activity its immunological properties were investigated further.

Precipitin titrations with the soluble antigens of vaccinia are regularly made by incubating the antigen-antibody mixtures in closed tubes at 50° C. for 18 hours.   In view of the experiments with concentrated filtrate described above, it seemed important to eliminate the possibility that component III had only L-activity and that during incubation at 50° C. the native material was degraded to a substance which had the mobility of component II and which now for the first time was capable of precipitating with S-antibody.   Serial dilutions of pure component III were set up with optimal amounts of L- and of S-antibodies and one pair of each of four such sets of titrations was incubated at 3°, 20°, 37°,

and 50° C.   In each set of titrations the precipitin endpoint in the presence of S-antibody was identical with that observed in the presence of L-antibody.   These findings clearly indicated that component III in its native state contained both L- and S-reacting portions.

Absorption experiments in which pure solutions of component III were treated with L-antisera showed that removal of all L-reacting material simultaneously removed all S-reacting material.   Similarly, absorption with S-antibody eliminated L- as well as S-precipitinogen.   The

Precipitin	L	Negative		1:1600
titers	S	Negative		1:1600

Precipitin	L	1:1600		1·128
titers	S	1:1600		1.128
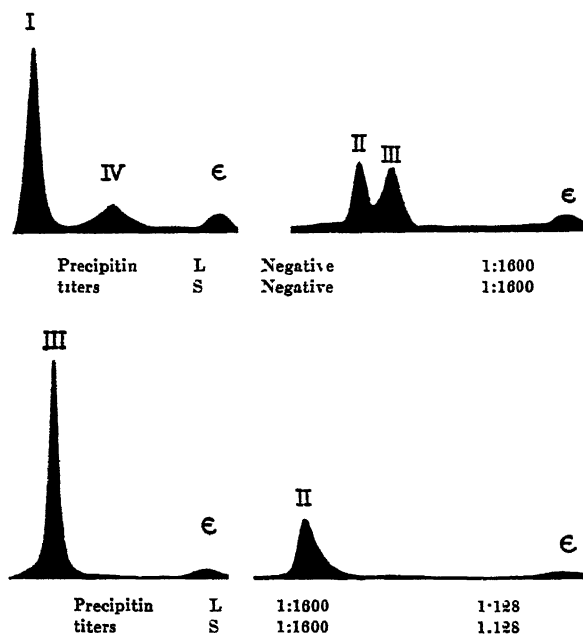
FIGURE 4.   Electrophoretic patterns of fractions from dermal filtrate.

results of a typical absorption experiment are summarized in TABLE 2; they demonstrate that the two serologically distinct parts of the molecule are inseparable in their native state.   This single substance possessing both L- and S-reactive parts will henceforth be called LS-antigen.

Since pure LS-antigen was now available it seemed desirable to determine whether the previously recognized degraded forms of L- and S-reacting material could be obtained by appropriate treatment of electrophoretically and ultracentrifugally homogeneous LS.   This was done as follows.   An unbuffered solution of LS that had a titer of 1:1600 with L- and with S-antibody was divided into two portions, one of which, A,

TABLE 2

ABSORPTION OF COMPONENT III WITH L- AND S-ANTIBODIES

Antigen	Antisera	Dilution of antigen						
		1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
Component III unabsorbed	L	+++	+++	+++	+++	+++	+++	+
	S	±	±	+++	+++	+++	+++	+
Component III absorbed with L-antibody	L	–	–	–	–	–		
	S	–	–	–	–	–		
Component III absorbed with S-antibody	L	–	–	–	–	–		
	S		–	–	–	–		

was heated at 70° C. for one-half hour, and another, B, was treated with sufficient NaOH to bring the concentration to N 20 and then heated at 56° C. for one and one-half hours, after which the pH was adjusted to 7.0. Solution A no longer precipitated with L-antibody but still had a titer of 1:1600 with S-antibody. Solution B precipitated with neither L- nor S-antibody. Inhibitory power of these preparations was investigated by adding varying amounts of solution A to constant quantities of L-antiserum, and of solution B to S-antiserum. After incubation at 50° C. for one-half hour the mixtures were tested in the usual way for precipitating antibodies. Some of the data obtained in this experiment are summarized in TABLE 3. It is apparent from these results that various levels of degradation of LS can be obtained, leaving either the L- or S-part of the molecule in a stage where it can combine with the corresponding antibody without precipitation. Since LS has been employed to represent the molecule in its native state it will be convenient to use the symbols L'S for the heat-altered antigen and L"S' for the substance obtained after heating in the presence of alkali. In FIGURE 5 the
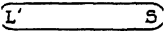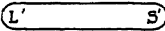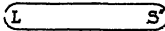
Serological activities of LS-antigen and some of its degradation products

Native	Heated	Heated with alkali	Digested with chymotrypsin
L̲ ⃝ S	L̲' ⃝ S	⃝ L' ⃝ S'	L̲ ⃝ S'
Precipitates with both L- and S-antibodies	No precipitation with L-antibody Inhibits L-antibody Precipitates with S-antibody	No reaction with L-antibody No precipitation with S-antibody Inhibits S-antibody	Precipitates with L-antibody No reaction with S-antibody

FIGURE 5.

serological activities of LS-antigen and of some of its degradation products are summarized.

Electrophoretic studies on solutions of L'S and L"S' show that both substances are electrically homogeneous but have mobilities which differ from each other and from that of LS. Thus, in 0.05μ veronal buffer solution, pH 7.9, LS has a mobility of 4.0 × 10^{-5} cm. 'sec. per volt cm., while L'S prepared from the same lot of antigen has a mobility of 5.9 × 10^{-5}. This fact is of particular interest for it clarifies an earlier perplexing observation. It is now apparent that L'S and serologically inert component II from dermal filtrate have nearly identical mobilities. L"S' examined electrophoretically under similar conditions has a mobility

of 6.4×10^{-5} cm./sec. per volt cm.   The mobility of this degradation product is close to that of component I in dermal filtrate; the two are not identical. however, for the latter fails to inhibit S-antibody.

TABLE 3

INHIBITION OF L- AND S-ANTIBODIES BY DEGRADED FORM OF LS-ANTIGEN

solution	Ratio of inhibiting solution to antiserum	Test anti-serum	Test antigen				
			1:8	1:16	1:32	1:64	1:128
None		L	++++	++++	+++	+++	++
		S	++++	++++	+++	+++	+-
LS heated	1:1	L	+	–	–	–	
LS heated with alkali	1:1 0.5:1	L S	++ –	++++ –	+++ –	++ –	

Recently, another degradation product of LS has been obtained[11]. This substance, prepared by digesting pure LS-antigen with crystalline chymotrypsin under proper conditions, precipitates with L-antibody but not with S-antibody; furthermore. it does not inhibit the latter.   This material has been designated LS″ (see FIGURE 5).   It represents the degradation form of LS which was postulated on theoretical grounds in earlier reports[9, 10] to account for certain experiments in which dermal filtrate treated with S-antibody was freed of all S-precipitinogen but still contained demonstrable L-precipitinogen.

Physical and chemical properties of LS and some of its degradation products have been determined and will be reported in detail in the near future.[12]   It may be briefly stated here that LS is characterized as follows: density $= 1.39$, specific volume $= 0.72$, diffusion constant $= 1.5 \times 10^{-7}$, sedimentation constant $= 4.3$ s (Svedberg units), all at $4°$ C.; the electrophoretically determined isoelectric point is pH 4.8. Chemical analyses show 15.7 per cent nitrogen but no lipid, nucleic acid. phosphorus nor glucosamine.   The LS-antigen appears to be an elongated protein molecule with a molecular weight of about 240.000 and with an axis ratio of approximately 30:1.

CONCLUSIONS

The virus of vaccinia has a complex structure since at least five antibodies develop in animals following infection or hyperimmunization with

active elementary bodies, *viz.*, a neutralizing antibody, an agglutinin designated X, antibody against a nucleoprotein constituent (NP) of the virus, and, finally, antibodies against a heat-labile (L) and heat-stable (S) soluble antigen. L- and S-antigens, although immunologically distinct, are not separate substances; they are component parts of a single substance, LS.

The LS-antigen of vaccinia provides a clear example of a single molecule which is capable of eliciting two distinct antibodies. It is theoretically possible that a third antibody, which is capable of reacting with both the L- and S-parts of the molecule, may also be elicited. However, the methods employed would not permit the identification of such an antibody.

Two levels of degradation of both the L- and of the S-parts of the LS-molecule have been demonstrated. In the first stage the property of precipitating with the corresponding antibody disappears but the power of inhibiting this antibody remains; in the next stage, serological activity is not demonstrable. It has thus been possible to prepare materials from LS-antigen which precipitate only with L- or only with S-antibody.

REFERENCES

[1] a Hughes, T. P., Parker, R. F., & Rivers, T. M. Jour. Exp. Med. **62**: 349. 1935. (b) McFarlane, A. S., MacFarlane, M. G., Amies, C. R., & Eagles, G. H. Brit. Jour. Exp. Path. **20**: 465. 1939. (c) Smadel, J. E., Lavin, G. I., & Dubos, R. J. Jour. Exp. Med. **71**: 373. 1940. (d) Hoagland, C. L., Smadel, J. E., & Rivers, T. M. Jour. Exp. Med. **71**: 737. 1940.

[2] Sternberg, G. M. Cent. Bakt. I Abt. Orig. **19**: 857. 1896.

[3] Craigie, J., & Wishart, F. O. Jour. Exp. Med. **64**: 819. 1936.

[4] Craigie, J., & Wishart, F. O. Jour. Bact. **35**: 25. 1935. Abstract.

[5] Smadel, J. E., Hoagland, C. L., & Rivers, T. M. Arch. Path. 1942, in press.

[6] a Parker, R. F., & Rivers, T. M. Jour. Exp. Med. **83**: 69. 1936. (b) Salaman, M. H. Brit. Jour. Exp. Path. **15**: 245. 1937.

[7] Parker, R. F., Jour. Exp Med. **67**: 361. 1938.

[8] Smadel, J. E., & Rivers. T. M. Jour. Exp. Med. **75**: 131. 1942.

[9] Parker, R. F., & Rivers, T. M. Jour. Exp. Med. **65**: 243. 1937.

[10] Shedlovsky, T., & Smadel, J. E. Jour. Exp. Med. **75**: 165. 1942.

[11] Smadel, J. E., Hoagland, C. L., & Shedlovsky, T. Jour. Bact. **43**: 96. 1942. Abstract

[12] Shedlovsky, T., Smadel, J. E., Rothen, A., & Hoagland, C. L. To be published.

PURIFICATION AND PROPERTIES OF THE PROTEIN OF THE "M SUBSTANCE" OF GROUP A HEMOLYTIC STREPTOCOCCUS*

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The M substance characterizes each of the various types of virulent Group A hemolytic streptococci, as is shown by specific precipitation of extracts of the M substance with appropriate antisera.[1] The typing obtained by the use of the M substance agreed with that previously obtained for the same strains by protection tests. More recent studies[2] also have identified the M substance with the substance in intact streptococci which stimulates the production of protective antibodies in animals. The active material isolated from the neutralized N 20 HCl extracts used for typing of the streptococci has been designated the M substance by Lancefield,[3] who also showed that its reactivity with antisera is rapidly destroyed by proteolytic enzymes. Although the type-specific substance thus was shown to be a protein, it did not give rise to antibodies when injected into rabbits. Stamp and Hendry[4] modified the isolation procedure, principally by using a milder extraction temperature, and obtained a fraction which induced active immunity in mice. Heidelberger and Kendall,[5] by extracting ground streptococci with increasingly alkaline solutions, isolated a fraction which produced type-specific precipitins when injected into rabbits. Their preparations contained phosphorus. Mudd and collaborators have used sonic treatment of the streptococci and neutral extraction to obtain a fraction with type-specific properties. In a recent paper[6] summarizing these studies it was concluded that two serologically active components were present and that the predominant component was a nucleoprotein of broad reactivity and the other was a type-specific material probably related to the M substance or the T substance recently described by Lancefield.[7] In the studies of Hirst and Lancefield[2] a substance was isolated by a modification of the original method of Lancefield which induced active immunity in mice and which in rabbits gave rise to precipitins and to protective antibodies passively transferable to mice. Considerable nucleic acid was present in their preparations in addition to protein. At the same time studies done in our laboratory[5] had shown that about a third of the material extracted at 56° C. with N 20 HCl was nucleic

acid. Our studies had also confirmed the extremely rapid inactivation produced by proteolytic enzymes and had shown that the M substance could no longer be precipitated with dilute acid from enzyme-treated solutions. We had shown further that the nucleic acid present is of the ribose type, and although the enzyme, ribonuclease, altered the acid-precipitability of the M substance it had no effect on its serological reactivity. It was concluded, therefore, that the nucleic acid did not contribute to the serological specificity of the M substance and that the active component was a protein.

In the present studies the relation of the protein and nucleic acid in the M substance has been investigated further. This work has culminated in the separation and purification of the type-specific protein. The course of this investigation and some of the properties of the type-specific protein are described. These studies were done with strains[a] 1048 and 1685 of virulent Group A hemolytic streptococci.

ISOLATION AND YIELD OF THE M SUBSTANCE[b]

Details of the isolation procedure used have been described.[3] A summary is given in TABLE 1. The amounts of the acid- and alcohol-precipitated fractions obtained have been about equal; the total of both fractions isolated has been 4.6 ± 1.3 (S. D.) per cent of the dry weight of the organisms extracted.[c] The first extracts are in most cases a golden yellow color; this is probably riboflavin as most of the color can be discharged with sodium hyposulfite. The point at which extraction is complete is strikingly demonstrated by the lack of clarity of the supernate obtained after centrifuging. While the M substance is being extracted from the organisms the supernates are clear; where extraction is complete even prolonged centrifuging does not give a clear supernate and the organisms do not pack as well. The small amount of material sometimes obtained in these turbid extracts is non-specific in its precipitation with antisera.

The acid-precipitated fraction, on the basis of phosphorus content

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[a] These strains in previous reports were designated Types 0 and 1 respectively and had so been typed in Dr. F. Griffith's laboratory.[9] Recent studies by Dr. Rebecca C. Lancefield and Dr. Alice C. Evans with strain 1048 have shown that it is not Type 6. There is no question of the virulence and the group classification of this strain but at present its type has not been definitely assigned. Reference to dry cultures prepared when the strain was received in this laboratory have led to the same conclusion. Dr. Lancefield has assigned the 1685 strain to Type 3. Dr. Griffith in reporting the type of this strain commented that in addition to colonies of Type 1, colonies were obtained which reacted with both Type 1 and 3 antisera. He had not yet decided whether the reaction with Type 3 antiserum was a group reaction. This does not entirely explain Dr. Lancefield's finding but it may be that in this strain both Type 1 and 3 antigens are present as Lancefield[10] has found for strain C203 although perhaps to a different or variable degree.

[b] The term, M substance, is retained for the material obtained from neutralized and dialyzed HCl-extracts by precipitation with acid, for historical reasons and also because to some degree it does represent a definite compound of protein and nucleic acid in salt-like union. The purified, active, protein component of the M substance is designated the M-protein.

TABLE 1

ISOLATION OF THE TYPE-SPECIFIC M SUBSTANCE FROM THE
GROUP A HEMOLYTIC STREPTOCOCCI

Undried, whole streptococci[1]
Approximately 2.0% (dry
weight) suspension in
N 20 HCl containing
2% NaCl; 16 hrs. at
56° C.

Centrifuge

Extract   Residue[2]

Neutralize and dialyze

Precipitate at pH
3–4 with N HCl

Precipitate: Supernate
M SUBSTANCE

                                                   Precipitate with
                                                   equal volume of
Dry with alco-   95% ethyl alcohol.
    hol and ether.

        Phosphorus
            content: 3–4%

            Precipitate
                                                   Supernate

        Dry with alcohol and ether.   discarded[3]

        Phosphorus content: 5–6%[4]

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[1] The organisms were grown 18 hours by a routine procedure which yielded about 0.2 gm. of dried streptococci per liter of culture medium. Recently, under the direction of A. M. Pappenheimer, Jr., the cultures have been kept neutral by the addition of NaOH during growth and yields of more than 1.0 gm. per liter obtained. Bernheimer, A. W., & Pappenheimer, A. M., Jr. Jour. Bact. In press. The yield of the M substance per unit weight of dry bacteria has been about the same from organisms grown in this manner as from those grown by the previous method.

[2] The Group A specific polysaccharide has been regularly isolated by us from this dried residue by Fuller's hot formamide method. The yields of polysaccharide have been about the same as with intact streptococci.

[3] Negligible amounts of material remain in this supernate. In Lancefield's original method for preparing extracts of streptococci, the Group A specific polysaccharide was isolated from this supernate. Her extractions, however, were made in a boiling water bath. Our extracts at 56° yielded only minute amounts of the group-specific polysaccharide.

[4] This fraction also contains M-protein. The ratio of protein to nucleic acid is about the reverse of that in the M substance. This is fractionated further with acid and alcohol into 3–4 per cent and 5–6 per cent phosphorus fractions. The former is essentially M substance and is added to it; the latter which is largely ribose nucleic acid, will be purified by methods suitable for nucleic acid. Considerable of this nucleic acid will dialyze through cellophane and some must have been lost in the preparatory dialysis.

(3-4 per cent), is about two-thirds protein and one-third nucleic acid, whereas in the alcohol-precipitated fraction (5-6 per cent phosphorus) this proportion is reversed.   On this basis about 2.5 per cent of protein has been obtained from the streptococcus.   Some part of this is not the M-protein as judged by the appearance of variable amounts of insoluble material in the purification.   However, it appears that this strain of the streptococcus contains about 2 per cent of the M-protein.

Although reprecipitation of a particular preparation of the M substance with acid at the point of optimum flocculation gave phosphorus values close to the original, precipitation of the same lot at two different pH values gave different phosphorus contents, with less at the higher pH.   These results suggested that a considerable amount of the nucleic acid was free and not firmly bound to the protein.[d]   This was confirmed by the electrophoretic data to be discussed later.

SEPARATION OF THE M-PROTEIN

The protein is precipitated from solutions of the M substance by half saturation with ammonium sulfate made neutral with ammonium hydroxide.   After being chilled, the precipitate is removed by centrifuging, and is dissolved in a small amount of water and dialyzed against 3 to 6 successive 2 liter portions of distilled water in the refrigerator for 24 to 48 hours.   Some of the protein precipitates during the dialysis.   Precipitation is completed by the careful addition of $N/10$ HCl.   When the nucleic acid content has been reduced to about 1.0 per cent, maximum precipitation occurs at pH 5.0.   With larger amounts of nucleic acid, the pH for maximum precipitation is at lower values.   In routine practice one volume of 95 per cent ethyl alcohol is added before adjustment of the pH, since in this case the point of maximum precipitation is much more striking.

The supernate from the ammonium sulfate precipitate is dialyzed and then precipitated by the addition of several volumes of 95 per cent ethyl alcohol and adjustment of the pH.   This fraction, designated "nucleic acid" in TABLE 2, still contains some M-protein.

The data obtained in numerous experiments with ammonium sulfate

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[c] When the extractions were performed with the stirring produced by convection currents obtained by partly immersing the vessel used in the 56° bath, 2 to 3 extractions were required to obtain all of the M substance.   By the use of thorough mechanical stirring all of the M substance can be obtained in one 16-hour extraction period; in many cases, one 6-hour extraction has been sufficient.

[d] Although the nucleic acid appears to be free, on the basis of chemical fractionation and electrophoresis, polar forces no doubt exist between the protein and nucleic acid and precipitates of the two represent a salt-like combination.   The term, protein nucleate, will be used to describe such combinations.   It will be seen that the M substance can be regarded as a protein nucleate.   The term nucleoprotein is used to designate compounds of nucleic acid and protein with non-polar bonds such that electrophoresis and chemical fractionation will not separate them.   The plant viruses are nucleoproteins in this sense.

are given in TABLE 2. A complete separation of protein and nucleic acid is not obtained in one precipitation. In successive precipitations the nucleic acid content of the protein fraction is reduced about 60 per cent each time. The small amount of nucleic acid remaining after the third precipitation does not represent a minimum. Further precipitations were not done because the preparations were satisfactory for the other

TABLE 2

SUMMARY OF PROCEDURE FOR SEPARATING M-PROTEIN AND NUCLEIC ACID
BY PRECIPITATING WITH AMMONIUM SULFATE; DISTRIBUTION
AND ANALYTICAL DATA FOR VARIOUS FRACTIONS

Procedure	Fraction (characteristic)	Recovery		Phosphorus content, %[3]
		%	% original[1]	
1. Combined original *acid* precipitated preparations.			100	3–4
2. Solution and reprecipitation with acid and alcohol.	Insoluble[2]	5	5	—
	Protein	65	65*	3.0
	Nucleic acid	20	20	6.5
3. Solution and precipitation of protein fraction of 2 with ammonium sulfate; recovery of precipitate and supernate.	Insoluble[2]	4	2.5	—
	Protein	40	26 *	1.0
	Nucleic acid	40	26	4.5
4. Repetition of 3 with protein fraction of 3.	Insoluble[2]	4	1	—
	Protein	70	18 *	0.3
	Nucleic acid	15	4	1.8
5. Repetition of 3 with protein fraction of 4.	Protein	85	15	0.15
	Nucleic acid	10	2	1.0

[1] The figures marked * in this column represent the portions that are reprecipitated. Addition of the other parts gives the amount recovered—75.5 per cent. The amount unaccounted for is largely nucleic acid lost by dialysis. In a number of cases, the original acid-precipitated preparations were combined, ground in a mortar for easier solution, and step 3—ammonium sulfate precipitation—was carried out at once. In this case, recoveries had been even lower in a summary of averaged data, 69 per cent). Analysis of the data showed the loss was in the high-phosphorus fraction corresponding to the "nucleic acid" of step 2, for which dialysis was not used. The dialyzability of the nucleic acid was shown by comparative data for weight recovery and phosphorus recovery. In every case, the latter was smaller, in several instances 60 per cent as compared to 80 per cent.

[2] The appearance of insoluble material with successive drying and solution should not be regarded as evidence of alteration of the M-protein due to drying with alcohol and ether, since in numerous fractionations no insoluble material appeared after the solution of the combined original preparations. Some of our best preparations have withstood frequent drying with alcohol and ether without obvious changes in solubility. In fact, our experience suggested that any preparation which would not dissolve after such drying to give a 1-2 per cent solution in N,/10 salt at neutrality was not pure. Another characteristic of a pure preparation is lack of color. This will be discussed later.

[3] The nucleic acid content can be obtained by multiplying these values by 10.5. It had previously been shown[5] that the phosphorus represents nucleic acid.

work to be described.   The final yield of M-protein after repeated pre-
cipitations is 15 per cent of the starting material.   This yield is increased
when the M-protein in the "nucleic acid" fractions is recovered by simi-
lar repeated precipitation.

Preparations of the M-protein obtained by this procedure contained
14.4 per cent nitrogen after drying in the vacuum oven at 50° C.   No
correction was made for the ash content which probably was small be-
cause prolonged dialysis was used.   Several preparations were homo-
geneous by electrophoresis and one preparation studied was homo-
geneous by sedimentation and diffusion.

OTHER METHODS TRIED FOR THE SEPARATION OF PROTEIN AND NUCLEIC ACID

Precipitation of the nucleic acid in the M substance with barium was
tried with no success.   A small amount of precipitate was obtained but
subsequent isolation of the protein revealed it still contained considerable
nucleic acid.

Another method tried for the separation of the protein and nucleic
acid was precipitation with glacial acetic acid.   It was hoped this would
be successful with the high nucleic acid-fractions.   High and low nucleic
acid-fractions were obtained by this means but the separation of protein
and nucleic acid was inefficient.

Ammonium sulfate precipitation at neutrality was used with the
nucleic acid, alcohol-precipitated fractions (see TABLE 1) but was not
practical because of the large loss of nucleic acid during dialysis.   In
several experiments only 50 per cent of the starting material was re-
covered.   In parallel experiments with yeast, more than 80 per cent of
nucleic acid was recovered.   This suggests that the molecular weight of
the nucleic acid in the streptococcal extracts was considerably less than
the 17,000 estimated by Loring[11] for yeast nucleic acid.   It seems un-
likely that the streptococcal nucleic acid was homogeneous in size be-
cause smaller amounts were lost with successive dialysis of this material.

The nucleic acid content of the M substance could be reduced by
warming the latter with N 100 NaOH, followed by neutralization,
dialysis and recovery of the protein.   This method was not used ex-
tensively because considerable loss of protein occurred.

INCREASE IN SOLUBILITY OF THE M-PROTEIN AT ELEVATED TEMPERATURE

In working with an aqueous, 0.2 per cent solution of the M-protein it
was observed that the flocculent precipitate obtained at pH 5.2 at room

temperature could be dissolved by warming at 56° C. On cooling this solution, the M-protein reprecipitated. Subsequently, it was found that neutral solutions of appropriate concentration would show this phenomenon also, *i.e.*, the solubility of the protein, either isoelectric or as a salt, was considerably enhanced by an increase in temperature. When warm saturated solutions of the M-protein were cooled the appearance of the precipitate differed markedly from that obtained by isoelectric or salt precipitation. The precipitate was apparently granular although it did not settle rapidly. Examination of this material in the dark field showed the presence of small spherical particles about 5 microns in diameter. When crushed they were seen to be solid but apparently soft. This may represent a stage of crystallization. However, repeated precipitation by this method has failed to give definite crystals. All preparations of the M-protein examined have behaved similarly.

ELECTROPHORETIC EXPERIMENTS

This part of the work has been carried out in collaboration with Dr. Florence Seibert of the Henry Phipps Institute.[12] The apparatus and technic developed by Tiselius[13] were used. Data obtained with the M substance are given in TABLE 3. In four of six preparations examined
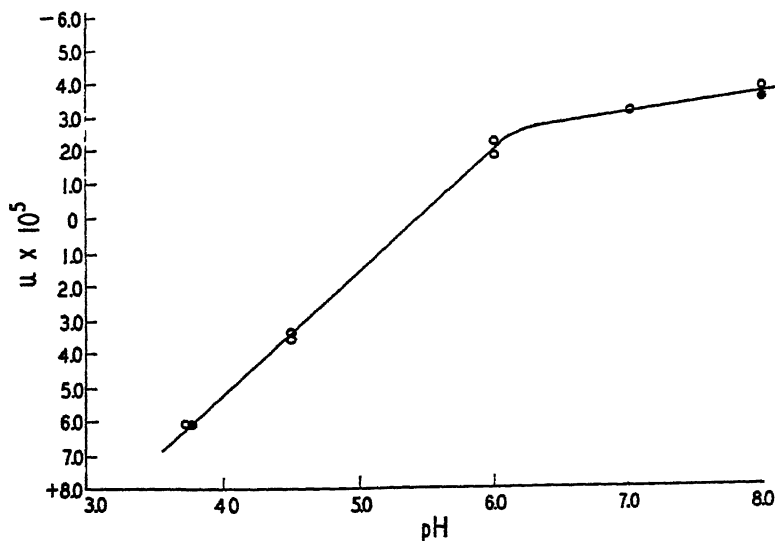
FIGURE 1. Mobility of the M-protein in acetate (pH 3.7 and 4.5) and phosphate pH 6.0, 7.0 and 8.0) buffers, μ 0.1. The mobility (u is in terms of cm.^{2} volt^{-1} sec.$^{-1}$.

only the two components mentioned were observed; in the other two a small amount of a third component with a mobility of −6.0 at pH 6 appeared. In several experiments the components described in the table were isolated. The results obtained with the preparation containing 2.3 per cent phosphorus shown first in the table are typical. The material isolated from the cell to which the fast component had migrated contained 7.7 per cent phosphorus. Thus, the fast component was identified with the nucleic acid. The material from the cell containing the slow component contained 1.1 per cent phosphorus and reacted type-specifically with homologous antisera. Subsequent work showed that the nucleic acid contained in the material in this cell was due to incomplete separation. Curves showing the mobility of the purified M-protein at various pH values are presented in FIGURE 1. The open circles represent preparations of the strain 1048 M-protein and the solid circles preparations of the strain 1685 M-protein. No difference was observed between the proteins of the two types studies. Also there was little or no difference in the mobility of the protein when purified or when admixed with nucleic acid as in the M substance.

TABLE 3

MOBILITY OF THE PROTEIN AND NUCLEIC ACID IN PREPARATIONS OF THE M SUBSTANCE

Preparation	Phosphorus content	Concentration, total, by weight	pH Phosphorus buffer (μ 0.1)	Mobility (u) x 10^5 cm.^{2} volt^{-1} sec.$^{-1}$, [1-4]	
				Protein	Nucleic Acid
Standard procedure except precipitation at pH 4.7.	2.3%	0.6%	6.0	−2.3	−11.4
Standard procedure and reprecipitation.[5]	1.1	0.5	6.0	−2.2	−10.3
Standard procedure and reprecipitation.	1.1	0.5	8.0	−3.8	−14.2
Prepared from organisms grown 4 hours.	3.4	0.25	8.0	−3.6	−14.3

[1] The current for these runs was 13.4 to 13.8 milliamperes, the potential gradient 5.85 to 7.12 volts cm.$^{-1}$. Part of the variation in the latter was due to the use of cells of different cross section.
[2] The mobilities given are for the descending boundary which has been shown to give the truer mobility, uncomplicated by ionic concentration effects.
[3] The sign designates the charge on the molecule, i.e., a negative sign indicates movement toward the positive pole.
[4] Throughout the text, mobilities are given as whole numbers; the dimensions are as indicated here.
[5] In the course of several reprecipitations, this preparation was warmed with N/10 NaOH and treated with ribonuclease[8] which probably assisted in reducing the nucleic acid content.

Electrophoretic examinations were made of other streptococcal fractions because of their possible relation to the M-protein. Some representative observations are given in TABLE 4.

Another protein-nucleic acid fraction (NPA)[14] that we have examined is obtained by neutral extraction of streptococci disintegrated with sonic vibrations.[6] In some respects, it appears to be similar to the "neutral-extracted nucleoprotein" described by Heidelberger and Kendall.[5] The material prepared as described[6] contains variable amounts of nucleic acid. Yields of 15 to 25 per cent of the dry weight of the streptococci have been obtained. The mobilities of the protein when present in the NPA and of the protein when isolated from the NPA and purified were the same and the serological properties of the two were similar. The mobility of the NPA-protein was considerably greater than that of the M-protein. Serological data suggested the presence of about 1 per cent of type-specific substance in NPA.[6] However, no M-protein was observed electrophoretically in any of the NPA preparations studied. It was also absent from an original sonic extract examined in 1.5 per cent concentration.

Some data are shown for purified streptococcal nucleic acids.[e] The mobilities of the nucleic acids are of similar magnitude to those observed in the mixtures of nucleic acid and protein as isolated. The values were somewhat lower than obtained in two experiments with yeast nucleic acid (-15 at pH 6.0, -19 at pH 8.0). Stenhagen and Teorell[15] have reported mobilities for thymus nucleic acid of -17.4 at pH 5.86 and -20 at pH 7.90 and μ 0.1.

The mobilities obtained for the vitreous humor polysaccharide are the same for the two pH values at which it was studied. A similar lack of change in mobility with change in pH has been reported for heparin.[17] The streptococcal capsular polysaccharide with chemical composition similar to the vitreous humor polysaccharide[18, 19] would be expected to behave similarly. No such component was observed in any of the streptococcal preparations examined.

The group-specific polysaccharide had negligible mobility and probably belongs to the group of "neutral" polysaccharides.[20] It was separated by electrophoresis from one of the nucleic acid preparations[16] and its identity confirmed by electrophoretic examination of material isolated by Fuller's formamide method[21, 22] from the acid-extracted residue (see TABLE 1).

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[e] These nucleic acids were prepared by Dr. M. G. Sevag and Mr. J. Smolens.[14]

TABLE 1

ELECTROPHORETIC DATA FOR OTHER STREPTOCOCCAL COMPONENTS

Preparation	Concentration, total by weight	Phosphorus content	Phosphorus buffer μ 0.1, pH	Mobility (u) × 10⁵ cm.² volt⁻¹ sec⁻¹		
				Protein	Nucleic acid	Other components
NPA	0.7%	3.6%	6.0	-6.2 -5.8	-11.7	-5.0; small amount
Principal protein component of NPA (P₂)[4]	0.2	<0.1	6.0			
NPA	0.9	1.5	8.0	-7.1	-16.3	-14; visible only on ascending side
Principal protein component of NPA (P₂)[4]	1.0	<0.1	8.0	-7.7		Immobile boundary (both sides), separated by compensation device; no ppt, with anti-serum
Nucleic acid (N₂) from NPA[1]	1.0	9.10[1]	6.0		-12.3	Immobile boundary separated, no activity with antisera
Nucleic acid (MZR) from sonic residue[1]	1.0	8.87[2]	6.0		-13.2	Immobile boundary separated, reacted with Group A streptococcus antisera. Separated nucleic acid reacted only with pneumococcus antisera.[16]
Nucleic acid (N₂) from NPA[1]	0.5	9.10[1]	8.0		-17.1	
Vitreous humor polysaccharide[3]	0.5		6.0			-10.4
Vitreous humor polysaccharide[3]	0.5		8.0			-10.3
Streptococcus Group A specific polysaccharide	1.0		7.0 (μ 0.02)			-0.2

[1] Contained 25 per cent thymus-type nucleic acid (diphenylamine reagent); the remainder presumably was yeast-type nucleic acid since positive color tests for yeast-type nucleic acid were obtained. This preparation was shown to be pure nucleic acid by its containing the theoretical amount of phosphorus and the equivalence of the phosphorus and purine nitrogen values.
[2] This preparation contained no thymus-type nucleic acid.
[3] This material, prepared from bull's eyes, was studied because the streptococcal capsular polysaccharide has been shown to be similar chemically.[18,19]
[4] Prepared by Dr. M. G. Sevag and Mr. J. Smolens.[11]

SEDIMENTATION AND DIFFUSION BEHAVIOR
OF THE M-PROTEIN

These experiments were performed by Dr. A. M. Pappenheimer. Jr.[*] in the laboratory of Physical Chemistry at the University of Wisconsin. Several sedimentation velocity experiments were performed using a standard Svedberg oil-turbine centrifuge from which a sedimentation constant (S_{20}) of 1.7 x 10^{-13} cm. sec. dyne was calculated. The scale average displacement curves obtained were still sharp at the end of these experiments (2.5 and 4.5 hours, at 60,000 r.p.m.) indicating that little diffusion had taken place and suggesting a high degree of dissymmetry for the sedimenting unit. No other components were observed by sedimentation and this homogeneity was confirmed by diffusion. A diffusion constant (D_{20}) of 4.2 x 10^{-7} cm.^{2} sec. was calculated from two diffusion experiments. With these constants and value of 0.77 for the partial specific volume, the molecular weight was calculated to be approximately 41,000. The frictional coefficient ratio $(f_{fo}$ calculated from these data was 2.2, which indicates an extremely elongated molecule with a ratio of major to minor axis of at least 20 to 1. This large axial ratio was confirmed by viscosity measurements. In neutral M 10 sodium phosphate at 22.5° C. the relative viscosity of a 1.00 per cent solution was 1.35, of a 2.00 per cent solution 2.00. The reciprocals of these values when plotted against protein concentration fall on a straight line through the origin. Treffers[24] recently showed the validity of this relation for numerous proteins. The axial ratio calculated from the viscosity data with Polson's[25] empirical equation relating viscosity and axial ratio was of the same magnitude as that given above.

SEROLOGICAL PROPERTIES OF THE
PURIFIED M-PROTEIN

The chemical work described in the previous sections was done with material isolated from two strains of streptococci, representing two different types: strain 1048, and strain 1685. Larger amounts of material were available from strain 1048 because of the better growth of this strain. In many cases preliminary work was done with the M preparations of this strain but in all of the chemical work parallel experiments were eventually done with material from the 1685 strain, and in no case was any significant difference observed.

In the precipitin test the 1685 M-protein gives considerable cross-reaction with 1048 streptococcal antisera. The M-proteins used in these tests were homogeneous by electrophoresis. When the M-protein is

treated with trypsin, the precipitation with heterologous antisera is lost just as rapidly as the type-specific precipitation. These observations are strong evidence for the reality of this cross-reaction because they show that contamination is unlikely and that the cross-reaction has the same lability to trypsin as the specific reaction. The nature of this cross-reaction was brought out by quantitative determinations of the amount of precipitate obtained at several dilutions. These data are presented graphically in FIGURE 2.[f] The milligrams of N shown are the amounts precipitated from 1.0 milliliter of antiserum. FIGURE 2 curve I gives
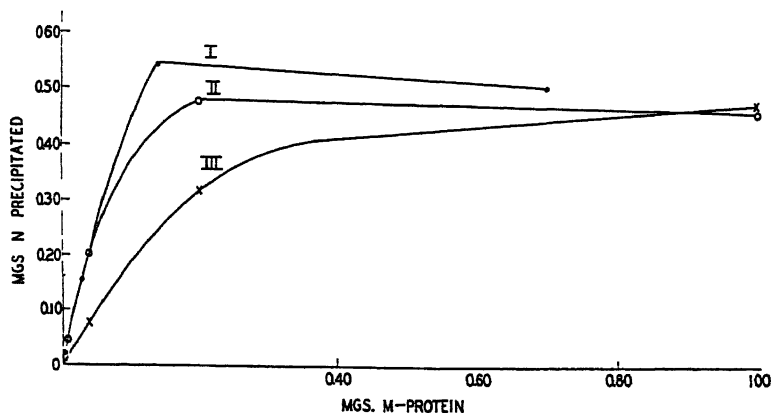
FIGURE 2. The precipitation of streptococcal antiserum (1.0 ml.) with homologous (I and II) and heterologous (III) M-protein.

data for the precipitation of a preparation of the 1048 M substance with 1048 antiserum and curve II precipitation of the purified 1048 M-protein with the same serum. The curve for the M substance is on the basis of its M-protein content. In curve III the precipitation of the 1685 M-protein with the 1048 serum is shown. All of these precipitations were performed with the same lot of pooled serum. The 1048 homologous antibodies of this serum could be completely removed by using an excess (1 mg. to 1 cc. of serum) of the heterologous 1685 M-protein. Neither the purified 1048 M-protein nor the 1048 M substance reacted with a group-specific antiserum[g] that reacted with dilutions of 1:2,000,000 of the purified group-specific polysaccharide. Hence both of these preparations must contain less than 0.1 per cent of the polysaccharide and

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[f] The point of inflection of curve I was confirmed by testing the supernates from the precipitates with antigen and antibody. The true point of inflection of curve II is slightly to the left of the point shown, since similar tests revealed a slight excess of antigen at this point.

[g] This antiserum was furnished by the Lederle Laboratories.

the presence of this polysaccharide can not explain the slightly larger amount of precipitate obtained with the M substance.

To complete the serological studies. experiments were performed in which preparations of the purified M-protein were used to precipitate streptococcal antisera that protected mice against relatively large doses of homologous streptococci.[h] The precipitates were removed and the protective power of the antisera tested again. These experiments were done in parallel. Dilute solutions of the 1048 M-protein (0.2 mg. in 1.0 ml. of 0.85 per cent NaCl was used to precipitate 1.0 ml. of antiserum, see curve II of FIGURE 2), were effective in removing the protective antibodies from antisera. An antiserum which, in 0.5 ml. amounts, protected a mouse against 10 minimal lethal doses (MLD) of streptococci, would no longer protect against 1 MLD.

Similar experiments made with the 1685 M-protein were not so successful, although a partial absorption of the protective antibody was obtained. The 1685 M-protein preparation used was not highly purified (it still contained about 10 per cent nucleic acid) and had not been studied in the electrophoresis apparatus. However, it represented a stage in the purification of a lot that in electrophoresis was a single substance and serologically it was a representative preparation, *i.e.*, it gave the cross reaction described with the 1048 antiserum and typical behavior with homologous 1685 antiserum. The preparation was type-specific (type 3) in its reactivity with absorbed antisera[i]. It may be that the rigorous treatment used in its purification, such as drying with alcohol and ether, etc., had partially denatured it so that some reactive groups remained but not its full potency as an absorbing antigen. The passive protection experiments were essentially specific although in a few experiments some cross-protection was obtained between the 1685 and 1048 strains. We have observed in active protection tests cross-protection between these same strains in that life was prolonged, but survival was not obtained as in the homologous test. These observations are in line with the cross reaction observed in the precipitin reaction (see FIGURE 2.).

DISCUSSION

The method of purification of the M-protein was designed to free it primarily of nucleic acid and secondarily of the small amount of other

protein which occasionally appears to be present. Preliminary tests have shown that the M-protein and the NPA-protein, which is the most likely contaminant of the former, are precipitated by about the same salt concentrations. Therefore, further purification by this means would be impossible or very difficult. Fortunately, extraction of the streptococci with dilute acid has considerable selectivity and only rarely was contamination other than nucleic acid observed in the M substance. The purified M-protein preparations, with one exception, have not shown any other definite component in electrophoresis although occasionally in electrophoretic observations made by the Svensson slit optical method the advancing limb of the concentration gradient-curve was unsymmetrical with respect to the other limb, suggesting the presence of small amounts of another component. The single exceptional preparation of the M-protein contained almost an equal amount of another component with the same mobility as the NPA-protein. In ten other M preparations examined no similar large contamination had been seen. This does illustrate, however, the need for a specific purification method. The observed increase in the solubility of the M-protein at higher temperature and subsequent precipitation on cooling seems to offer the desired specificity. Work in progress has shown already that the dark brown color accompanying some of the preparations can be eliminated by this means.

Some of the properties previously described for the M substance are of interest in view of the knowledge that it is a protein nucleate.[d] One of these interesting properties is its inability to precipitate with acids after a very brief treatment with proteolytic enzymes. One can conclude that brief digestion of the protein has produced units so small and soluble that nucleic acid will not precipitate them. A number of other proteins have been tested with nucleic acid before and after proteolytic digestion but in no case has a similar complete loss of precipitation been observed.

The form in which the M-protein occurs in the streptococcus is of great interest. The following evidence strongly supports the conclusion that it is very likely in a form different from that which we have described: (a) Little or nothing can be extracted from the streptococci by neutral solvents, whereas the M-protein isolated from the acid-extracts is quite soluble in neutral solvents. (b) The M-protein has been clearly related by absorption experiments to the protective antibodies produced with streptococcal vaccines. However, the M-protein produces very little protection when injected into animals. The protection obtained, although definite, is much less than that given by streptococci containing an equivalent amount of M-protein. (c) The physical data obtained for

the M-protein which show the ratio of major to minor axis is about 20 to 1 suggest, in the light of our knowledge of other proteins, that the protein as prepared may be in denatured form. If this is a denatured protein it has some properties usually not found in the denatured form, namely, its considerable solubility and also its excellent reactivity with antibodies, presumably to the native form of the M-protein since they were obtained with intact streptococci. However, it may represent a denatured protein or a part of a larger antigenic unit. The evidence presented in (a) and (b) would be compatible with the latter hypothesis. The appearance of nucleic acid in the acid-extracts that otherwise appear to be specific for the M-protein suggest a probable association of these two substances in the streptococcus.

We have been impressed with the apparent specificity of dilute acid in the extraction of the M-protein and its concomitant nucleic acid. To summarize, (a) electrophoretic examination of the M substance has shown it to be almost exclusively M-protein and nucleic acid; (b) the total yield of M substance is about the same whether extraction is carried out at 56° or 37° C.; (c) negligible amounts of the group polysaccharide are found in the acid extract of whole streptococcal cells whereas treatment of the acid-extracted residue by Fuller's method has yielded the same amount of this polysaccharide that exists in intact streptococci; (d) the nucleic acid in these acid extracts is always of the ribose type. The desoxyribose type is present in the streptococci also and has been extracted after sonic disintegration of the cells.

All of this suggests a specific extraction by acid of a particular part of the streptococci. perhaps a component of the cell-wall[26, 27] or part of it. We have other evidence also for the localization of the M-protein. Sonic treatment makes possible solution of 15 to 25 per cent of the streptococci by neutral solvents but only negligible amounts of the M-protein are present in solutions obtained by this procedure. This suggests that perhaps the M-protein is a part of the cell-wall which is disrupted but not brought into solution by this treatment. What seems to be direct evidence for this is obtained in electron microscope observations of sonically treated streptococci.[26] "Shadow" cells are seen which result from the loss of considerable electron absorbing material, probably the cell contents, since the size and shape of the cell are retained. The evidence we have given suggests a localization on the cell-wall. Other evidence suggests it is probably only a part of the bacterial cell-wall. Dr. Lancefield, investigating the action of trypsin on living streptococci, has observed that streptococci in cultures treated with trypsin remained viable but attempts to isolate the M substance from them

were unsuccessful.[23] This evidence and the studies of streptococcal variants strongly suggest that the M-protein, although a part of the cell-wall, may be highly specialized, having a protective role against phagocytosis[j] but not being otherwise necessary for the viability of the streptococcus.

[j] Virulent strains of streptococci have been shown to be less readily phagocyted than non-virulent variants of the same strain; combination of components of the bacterial surfaces with homologous antibodies renders the bacteria susceptible to phagocytosis.[6]

REFERENCES

[1] Lancefield, R. C.   Jour. Exptl. Med. 47: 91.   1928.
[2] Hirst, G. K., & Lancefield, R. C.   Jour. Exptl. Med. 69: 425.   1939.
[3] Lancefield, R. C.   Jour. Exptl. Med. 47: 469.   1928.
[4] Stamp, T. C., & Hendry, E. B.   Lancet. 1: 257.   1937.
[5] Heidelberger, M., & Kendall, F. E.   Jour. Exptl. Med. 54: 515.   1931.
[6] Mudd, S., & Lackman, D. B.   Jour. Immunol. 39: 495.   1940.
[7] Lancefield, R. C.   Jour. Exptl. Med. 71: 521.   1940.
[8] Zittle, C. A.   Jour. Immunol. 37: 1.   1939.
[9] Mudd, S., Czarnetzaky, E. J., Lackman, D. B., & Pettit, H.   Jour. Immunol. 34: 117.   1938.
[10] Lancefield, R. C.   Jour. Exptl. Med. 71: 539.   1940.
[11] Loring, H. S.   Jour. Biol. Chem. 128: 61.   1939.   (abstract)
[12] Zittle, C. A., & Seibert, F. B.   Jour. Immunol. 43: 47.   1942.
[13] Tiselius, A.   Trans. Faraday Soc. 33: 524.   1937.
[14] Sevag, M. G., Lackman, D. B., & Smolens, J.   Jour. Biol. Chem. 124: 425.   1938.
[15] Stenhagen, E. & Teorell, T.   Trans. Faraday Soc. 35: 743.   1939.
[16] Lackman, D. B., Mudd, S., Sevag, M. G., Smolens, J., & Wiener, M.   Jour. Immunol. 40: 1.   1941.
[17] Wilander, O.   Scand. Arch. Phys. 81: (Suppl. 15).   1938.
[18] Meyer, K., Dubos, R. J., & Smyth, E. M.   Jour. Biol. Chem. 118: 71.   1937.
[19] Kendall, F. E., Heidelberger, M., & Dawson, M. H.   Jour. Biol. Chem. 118: 61.   1937.
[20] Meyer, K.   Cold Spring Harbor Symposia. 6: 91.   1938.
[21] Fuller, A. T.   Brit. Jour. Exptl. Path. 19: 130.   1938.
[22] Zittle, C. A., & Harris, T. N.   Jour. Biol. Chem. 142: 823.   1942.
[23] Pappenheimer, A. M., Jr., Williams, J. W., & Zittle, C. A.   Jour. Immunol. 43: 61.   1942.
[24] Treffers, H. P.   Jour. Amer. Chem. Soc. 62: 1405.   1940.
[25] Polson, A.   Kolloid Zeit. 88: 51.   1939.
[26] Mudd, S.,& Lackman, D. B.   Jour. Bact. 41: 415.   1941.
[27] Mudd, S., Polevitzky, K., Anderson, T. F., & Chambers, L. A.   Jour. Bact. 42: 251.   1941.
[28] Lancefield, R. C.   Harvey Lectures 36: 251.   1941.

COMPLEMENT*

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INTRODUCTION

Complementary activity has been studied for more than forty years but complement has defied all attempts at isolation.[1]   Its properties are only partially known and its structure has not been determined.   Indeed, Brocq-Rousseu and Roussel[2] recently stated that "complementary power comprises all the activities of unheated serum."

Complement is usually defined as the non-specific portion of fresh serum which is not increased by immunization, and which when added to sensitized cells results in their destruction.   In other words, *complement is still defined in terms of its function and not in terms of its constitution*.

The power of complement to hemolyze sensitized red blood corpuscles is only one of its many properties.   Among others are: (1) the lysis of certain sensitized bacteria;[3] (2) the opsonization of certain bacteria;[4] (3) the activation of thermostable immune opsonins;[5] (4) the acceleration or augmentation of the aggregation of certain antigens by their homologous antisera, whether the antigen is a bacterial suspension,[6] a protein in solution,[7] or a suspension of erythrocytes;[8] and (5) the capacity to kill bacteria (bactericidal action) in the absence of bacteriolysis.[9]

It has been claimed that there is evidence showing that (6) a thermostable constituent of complement operates in the destruction of viruses by immune sera.[10]   It has been postulated that (7) a toxin-antitoxin complex binds complement *in vivo*,[11] and that by this mechanism the toxin is neutralized.   Furthermore, (8) complement has been said to be associated with the coagulation processes of blood,[12, 13] but recent evidence appears to invalidate this contention.[14, 15, 16]   (9) There seems to be a relationship between the sedimentation rate of erythrocytes and complementary activity,[17] *i.e.*, a fast sedimentation rate was found to be related to a low complementary activity.   (10) Complement has even been claimed to be associated with muscle contraction.[18]

It should be pointed out that complement, because of its multiple components, may be found to promote one of the aforementioned functions while failing to promote others; *e.g.*, a deficiency of one or more of

the components of complement may prevent hemolysis without affecting phagocytosis. Therefore, any activity of complement is determined by the substrate upon which it operates; and it thus becomes necessary in defining complementary activity or titer to define also the substrate employed. In this paper, unless otherwise noted, the substrate referred to is sheep red corpuscles sensitized with rabbit anti-sheep red cell serum.

At various times it has been suggested that complement is a simple chemical agent like oleic acid,[19] a lypolytic enzyme,[20] a proteolytic enzyme,[21] a catalyst,[22] a peptidase,[23] or more commonly a physicochemical state or a collodial attribute of fresh serum.[1] These theories, however, do not explain the fact that complement can be separated into two or more functionally distinct components, each of which is essential for the exhibition of its activity.

GENERAL PROPERTIES OF COMPLEMENT

Complement deteriorates rapidly with age. It loses its activity in a few days at $0°$ C., in a few hours at room temperature, and in a few minutes at $56°$ C. Hydrogen-ion concentrations acid to a pH of 5 and alkaline to a pH of 9 readily inactivate complement.[24] The activity of complement is inhibited by both hypotonic and hypertonic salt solutions, and especially by divalent ions.[25, 26] It is destroyed by shaking,[27] ultraviolet light[28] and proteolytic enzymes.[29] The optimum activity of complement occurs at a pH of 7.2–7.4 in physiological salt solution (0.9 per cent NaCl). Complement may be preserved for months by the process of freezing, drying and storing *in vacuo*.[30]

The narrow range of conditions under which complement is stable has contributed largely to the failure of earlier attempts at isolation.

PAST KNOWLEDGE CONCERNING THE COMPOSITION OF COMPLEMENT

It is known that treatment of serum with distilled water,[31] carbon dioxide[32] or dilute HCl[33] separates it into two thermolabile fractions which individually have no complementary activity; when combined, almost full activity is restored. The soluble fraction has been designated as the "albumin fraction" or the "end-piece," while the water-insoluble fraction has been termed the "globulin fraction" or the "mid-piece."

It has also been shown that yeast inactivates a relatively heat-stable component of complement, namely the "third component;"[34] while dilute ammonia destroys another fraction known as the "fourth com-

TABLE 1

RESUMÉ OF PAST KNOWLEDGE CONCERNING THE CONSTITUTION OF COMPLEMENT

COMPLEMENT

- Dilution and acidification
  - Precipitate
    - "Globulin fraction" or "mid-piece" is:
      1. Destroyed at 56° C. for 15 minutes;
      2. Unstable at room temperature.
  - Supernatant
    - "Albumin fraction" or "end-piece" is:
      1. Destroyed at 56° C. for 15 minutes;
      2. Stable in ice-box for at least 48 hours.
- Treatment with yeast cells
  - Removal of third component
    - The third component is:
      1. Destroyed at 66° C. for 30 minutes;
      2. Destroyed by cobra venom.
- Treatment with dilute ammonia
  - Removal of fourth component
    - The fourth component is:
      1. Destroyed at 66° C. for 30 minutes;
      2. Associated with bound serum calcium (?);
      3. Associated with lipids (?).

ponent."[35] Claims have been made for the occurrence of other components, but definite evidence of the existence of these is lacking.[1, 36] A schematic diagram of the components and their properties is given in TABLE 1.

THE ASSAY OF HEMOLYTIC COMPLEMENT

Three methods are commonly used in the quantitative estimation of complement in hemolysis:

1. The serial tube method is most widely employed. This method determines the smallest amount of serum necessary to cause complete hemolysis of a standard amount of sensitized red blood cells. The reciprocal of this minimum quantity is then assumed to be the complement titer in units.

Unless all procedures are controlled by careful standardization of the concentration of erythrocytes, by accurate estimation of the potency of the hemolysin employed, regulation of time and temperature, elimination of the effect of naturally occurring antibodies, correction for possible experimental variations and the degree of resistance of different red blood cells to hemolysis, serious errors may obscure otherwise significant results.

2. An alternative method which is often used measures the degree of hemolysis produced by a known amount of complement acting on a constant volume of red cells until the action is complete. This is known as the method of 50 per cent hemolysis.[37, 38] However, the same meticulous care must be employed in this method as in the serial tube method.

3. The observation that the complementary action of a given serum may be taken as a coefficient of the time required to produce complete hemolysis has led to the employment of another method.[39] The length of time needed to hemolyze a constant volume of sensitized corpuscles is assumed to be inversely proportional to the titer or activity of the complement.[40]

From the numerous criticisms which have been levelled at the available methods of complement titration[1] it is obvious that improvements are desirable. The present authors have availed themselves of the observation that the smallest amount of serum necessary to initiate hemolysis of a standard unit of red cells may be taken as a basis of comparison.[41] As most of the experiments detailed below involve the use of this method, the exact procedure employed in this laboratory is given.

Brooks' physiologically balanced salt solution, or in some instances when a buffered salt solution is undesirable, 0.9 per cent saline solution, is the diluent employed. This is previously boiled for one hour and

brought up to volume. The erythrocytes are obtained daily from healthy sheep from which the blood is drawn under aseptic conditions. The blood of the same animal is used throughout the course of any given experiment. One volume of cells is washed four times in ten volumes of either Brooks' solution or 0.9 per cent saline, and standardized to about 500,000,000 corpuscles per cc. Generally the cells are prepared just before the experiment, and are kept not longer than 12 hours so that errors due to increased cell fragility are avoided. Five units of a rabbit anti-sheep hemolysin of a titer of at least 1:4000 are used to sensitize the cells. After mixture of cells and hemolysin, the sensitized cells are allowed to stand at room temperature for about 15 minutes prior to use. The final mixture contains 250,000,000 erythrocytes per cc.

The blood to be tested for complementary activity is allowed to clot, and the clear serum immediately separated by centrifugation, care being taken to avoid hemolysis of the red blood cells. In no instance should this take over two hours from the time of bleeding. The serum is immediately diluted with the salt solution, 1:30 in the case of guinea pig serum, and 1:15 in the case of human serum; after dilution the serum is immediately titrated.

A series of 15 serological test tubes (acid-cleaned and freed of the last traces of acid with doubly-distilled water) is set up, and the diluted complement is carefully added in amounts ranging from 0.01 to 0.15 cc. with a 0.1 cc. pipette graduated in hundredths. Salt solution is then added to each tube so as to make a final volume of 0.15 cc. in each tube. One cc. of the standardized and sensitized cells is then added to each tube with rotation of the tube. The tubes are then well shaken by hand, and incubation is carried out in a water bath at 37° C. for 30 minutes. The tubes are then centrifuged at 1800 r.p.m. for 5 minutes; and the tube showing the first sign of hemolysis usually a yellow-orange color, *not red*) is taken as the initial point of hemolysis. With the normal guinea pig serum this is seen at about 0.02 cc. of a 1:30 serum dilution, while in human serum it generally occurs at 0.02-0.03 cc. of a 1:15 serum dilution. A control tube, containing only one cc. of the standardized cells plus 0.15 cc. of salt solution, should be carried through as above, so as to avoid errors indicative of cell fragility. The advantages of this method are obvious. Cell degradation products capable of inhibiting the function of complement are avoided. The use of an excess of amboceptor eliminates the error incurred by the natural hemolysin (anti-sheep hemolysin). Only a small amount of serum is needed for this test; and finally, the complement is allowed to operate according to its concentration.

By the use of this method, and avoiding all variations of procedure, results are obtained with an accuracy of ± 5 per cent.

THE PREPARATION, SPECIFIC INACTIVATION, AND REACTIVATION OF THE COMPONENTS OF COMPLEMENT

An improved method for the separation of the mid-piece and end-piece by the carbon dioxide method follows. By the use of this procedure little or no denaturation occurs in either of these two components.

Distilled water is saturated at room temperature with pure carbon dioxide gas which is allowed to bubble through the water for 30 minutes under slight pressure. One cc. of serum is added to 9 cc. of the carbon dioxide-saturated water, and carbon dioxide gas is again passed through the mixture for 20 minutes. Foaming produced by the bubbling of the gas is reduced by the addition of capryl alcohol with a wooden applicator stick dipped in the alcohol. Care should be taken to avoid an excess of alcohol because of its hemolytic quality. The globulin precipitated after 20 minutes saturation of the serum with carbon dioxide is rapidly centrifuged in an angle-centrifuge at room temperature at a speed of 4000 r.p.m., and resuspended three times in distilled water, after each time again being centrifuged as above. The euglobulin precipitated in the fractionation of one cc. of serum is then redissolved in 10 cc. of saline. and the residual carbon dioxide is taken off *in vacuo*.

The end-piece, which is present in the supernatant after the precipitation of the mid-piece, is made isotonic with 17 per cent NaCl solution. and the carbon dioxide is removed *in vacuo*. Both fractions are then tested for neutrality with brom-thymol blue. Each fraction should be inactive by itself, but almost fully active when combined with the other.

Although dilute ammonia is commonly used for the specific inactivation of the fourth component, it has been the practice in this laboratory to use hydrazine[42] instead of ammonia. It has been shown that at a pH of over 9 the mid-piece and the third compenent are unstable.[43] Furthermore, it was found that hydrazine inactivates the fourth component without any ill-effects on the other components. Therefore, to inactivate the fourth component of complement specifically, 0.15 cc. of 0.16 M hydrazine is added to one cc. of serum, thoroughly mixed and incubated in the water bath at $37°$ C. for one hour. The pH of such a mixture is 7.2-7.4. After incubation it is diluted with 9 cc. of 0.9 per cent NaCl solution.

Fresh yeast. or a yeast-powder (zymin), is usually employed to remove or inactivate the third component specifically. However, several workers have reported that the end-piece and the mid-piece are also

destroyed by yeast or zymin.[44]   In this laboratory the third component is usually inactivated by an insoluble carbohydrate recently isolated from fresh yeast.[45]   With this agent, *only* the third component is removed, and no inhibition due to diffusible substances occurs.   The method is as follows: 10 to 15 mg. of the insoluble carbohydrate is boiled for 30 minutes in 10 cc. of 0.9 per cent saline, centrifugalized, and the supernatant decanted.   One cc. of serum is added to the carbohydrate sediment, shaken, and incubated for two hours.   The mixture is further shaken every 30 minutes.   After the two hour incubation 9 cc. of 0.9 per cent saline is added, the contents mixed, and then centrifugalized until clear.   The supernatant is decanted, and contains all the fractions of complement with the exception of the third component.

As to the procedure of reactivation, it is the practice here to employ quantities of the components or inactivated complements identical with the smallest amount of the untreated serum needed to cause *complete* hemolysis of the standard unit of red blood corpuscles employed.   For reactivations the initial point method of hemolysis is not employed because more concentrated serum components are needed and these in turn interfere with the reading of initial hemolysis.

The inactivated complements or components are combined, incubated at 37° C. in the water bath for 15 minutes, and the solutions titrated for complementary activity.   The complementary titer is compared with that of an equal amount of the untreated serum and this is usually expressed in percentage of the original titer of the serum complement.

NEWER KNOWLEDGE OF THE COMPONENTS OF COMPLEMENT

All attempts to isolate complement or its components have hitherto failed.[5, 44]   Several investigators have tried to fractionate whole complement and its components with neutral salts such as ammonium sulfate.[46, 47, 48]   The results are discordant and too indefinite to characterize any one of the components as a functionally distinct entity.   Other methods, such as adsorption, extraction, and fixation have also proved to be fruitless.

It became apparent that although a great deal is known regarding the general properties of whole complement, little or nothing has been learned concerning the properties of its individual components.   Therefore it is clear that before isolations are attempted, the optimum conditions for their stability and activity should be ascertained.   Studies in this direction were made and the results of these experiments follow.

VARIATIONS IN GUINEA PIG COMPLEMENT

Because of its high content of hemolytic complement guinea pig serum was employed in all of the experiments described below. However, guinea pigs show wide seasonal variation in the complementary activity of their sera.

Several investigators[46, 50, 51] stated that complement suffers a reduction in titer during the winter months, and they attributed the seasonal variation to a deficiency in green foods and to a lack of vitamin C. Again, certain investigators[52] attempted to discover whether or not vitamin C entered into the constitution of complement since ascorbic acid does not exist in a free state in the blood. They claim that it is bound to proteins or lipoids but no proof is given for this statement. They found that the complementary power of the serum of rabbit was increased by the administration of vitamin C, and that a proportionately low value of vitamin C occurred in the adrenals of guinea pigs in which low complement titers were found.[52, 53] Later it was shown that a correlation exists between the complement titer and ascorbic acid content of guinea pig serum.[41] A similar qualitative relationship was also shown to occur in man.[54, 55, 56,§] Since these findings were made, it has been the practice in this laboratory to maintain all guinea pigs on a well-balanced diet supplemented with at least 10 mg. of ascorbic acid daily.[57]

It has also been noted that the action of whole native complement is associated with a redox mechanism.[57] The nature of the effect of vitamin C *in vivo* and of the redox mechanisms *in vitro* are suggestive, but as yet there is no explanation for either phenomenon. It would appear that only the third component is implicated in these phenomena. Also it is the opinion here that the vitamin C saturation is an index of an animal's well-being, and that the observed increase in complement in guinea pigs on high vitamin C diets is indicative of either the availability or the state of those serum entities responsible for complementary activity.

THE EFFECT OF VARIOUS HYDROGEN ION CONCENTRATIONS ON THE COMPONENTS OF COMPLEMENT

Although it is generally agreed that a pH under 5 and over 9 inactivates complement, nothing was known concerning the effect of pH

§ Crandon, Lund & Dill, (New England Jour. Med. **223**: 353. 1940), in a controlled study of vitamin C deficiency in a human reported that no relationship existed between the ascorbic acid content and complementary titer of blood plasma. However, the late date (78 days after onset of deficiency) on which the complement titrations were started and the possible technical differences of methods employed may account for this result. A larger series of cases with strict conformance to standardized technique would be desirable.

on the individual components of complement. The present authors
were able to show that end-piece and the fourth component are relatively
unstable in acid solutions and stable in alkaline solutions; and that the
mid-piece and the third component of complement are relatively un-
stable in alkaline solutions and more stable in acid solutions. This is
illustrated in TABLE 2. It is noted that after treatment of serum with
0.1 N NaOH to a pH of 9.5 the selective reactivation is brought about
only by ammonia-treated serum and the intact mid-piece. This in-
dicates that both the third component and the mid-piece are inactivated
at this alkaline pH. On the other hand, the treatment of serum with
0.1 N HCl to a pH of 4.2 resulted in its selective reactivation by zymin-
treated serum and the intact end-piece. Therefore, acids attack the
fourth component and the end-piece. These observations aided greatly
in the final separation of the components of complement from whole
serum as is discussed subsequently.

THE EFFECT OF AMINO COMPOUNDS ON THE
COMPONENTS OF COMPLEMENT

Although it was demonstrated in 1926 that dilute ammonia destroys
the fourth component of complement,[35] the nature of this inactiva-
tion was not explained. It has been postulated that the inactivating
process is due to the formation of an un-ionized calcium-ammonium
double salt.[35] Attempts have also been made to associate this in-
activation with lipids and bound calcium.[58, 59] The present authors in
collaboration with Dr. J. Seifter[42] formulated the hypothesis that the
ammonia-inactivation of the fourth component is the conversion of the
active carbonyl groups of the component to some less active structure.
To test this hypothesis, various amino compounds of two general classes
were tested for their anti-complementary effects: those amino compounds
which are known to react with carbonyl groups, and those which do not.
The results are summarized in TABLE 3. It is noted that (1) only pri-
mary amines, hydrazine, phenyl hydrazine and α-methyl hydroxylamine
specifically inactivate the fourth component; (2) simple alkalinity of
amines is not the cause of the inactivating property; (3) the fourth com-
ponent is inactivated only by amino compounds that are known to have
decided reactions with aldehydes; (4) the $-NH_2$ group is necessary for
this inactivation; (5) the type of substituent group replacing a hydrogen
atom of ammonia to produce the amino compound has a pronounced
influence on the capacity of the compound to inactivate the fourth com-
ponent; (6) amino compounds which have been previously treated with
aldehydes do not affect the fourth component; and (7) polar groups

TABLE 2

THE EFFECT OF VARIOUS HYDROGEN ION CONCENTRATIONS ON THE COMPONENTS OF COMPLEMENT

Complement treated with:	pH*	Per cent. hemolysis**	Reactivations with:						
			Heated serum	NH₄OH serum	Zymin serum	Mid-piece	End-piece	Heated mid-piece	Heated end-piece
			Percentile restoration of complementary activity						
NaOH (0.1 N)	10.4	0							
NaOH (0.1 N)	10.0	0	0	0	0	0	0	0	0
NaOH (0.1 N)	9.5	10	0	0	0	10	0	0	0
NaOH (0.1 N)	9.2	60	15	100	0	100	5	15	10
NaOH (0.1 N)	8.8	90	60	100	20	100	15	30	60
NaOH (0.1 N)	8.4		100	100	100	100	60	90	
Untreated	7.1	100							
HCl (0.1 N)	6.1	90	90	60	100	40	100	40	80
HCl (0.1 N)	4.9	40	30	15	85	10	100	10	20
HCl (0.1 N)	4.5	10	20	0	75	0	60	0	0
HCl (0.1 N)	4.2	0	0	5	0	0	0	0	0
HCl (0.1 N)	4.0	0	0	0	0	0		0	0
HCl (0.1 N)	3.5								

* Adjusted to 7.2 before titration.
** Titer of original serum taken as 100 per cent.

TABLE 3*

THE EFFECT OF AMINO COMPOUNDS ON THE FOURTH COMPONENT OF COMPLEMENT

Substituent type	Reagent	Formula	Minimal molarity	Maximal molarity	pH	Inactivation of fourth component
Positive	Ammonia	$H-NH_2$	0.16	—	8.5 10	—
	Methylamine	$CH_3 \ NH_2$	0.12	0.24	7.1 8.0	-
	Dimethylamine	$(CH_3)_2 \ NH$		0.72	7.0 10	-
	Trimethylamine	$(CH_3)_3 \ N$		0.72	7.0-10	-
	Tetramethyl ammonium hydroxide	$(CH_3)_4 \ NOH$		0.96	7.0 10	
	Ethylamine	$CH_3CH_2-NH_2$	0.12	0.24	8.0-9.0	+
	Diethylamine	$(CH_3CH_2)_2 \ NH$		0.72	7.0 10	-
	Triethylamine	$(CH_3CH_2)_3 \ N$		0.72	7.0 10	-
	Hydrazine	$H_2N \ NH_2$	0.08	—	7.2 8.0	±
Negative	Phenylhydrazine	$C_6H_5NH \ NH_2$	0.21	0.36	7.0 7.1	+
	Aniline	$C_6H_5 \ NH_2$		0.61	7.0 10	·
Polar	Urea	$NH_2CO \ NH_2$		0.95	7.0 10	·
	Acetamide	$CH_3.CO \ NH_2$				
	Glycine	$COOH \ CH_2 \ NH_2$		0.96	7.0 10	—
	Semicarbazide	$H_2N.CO.NH \ NH_2$		0.72	6.0 10	
	Thiosemicarbazide	$H_2N.CS.NH \ NH_2$		0.72	7.0 10	
	Hydroxylamine	$HO \ NH_2$		0.72	6.0 10	
Blocked polar	α-methyl hydroxylamine	$CH_3.O \ NH_2$	0.16	0.21	8.5 9.5	—
Indifferent	Methenamine	$(CH_2)_6 \ N$		0.96	7.0 10	·

Taken from **Pillemer, L., Seifter, J., & Ecker, E. E.** Jour. Immunol. **40:** 89. 1941.

which potentially lend acidic or redox properties to amino compounds completely abolish the anti-complementary effect of the amine. Blocking of the polar group restores this activity, as in the case of α-methyl hydroxylamine. In addition, amines which have negative and polar groups apparently need extra-physiological conditions of pH and temperature in order for the -NH₂ group to interact with aldehydes.

These results suggest that the active amino compounds act upon a carbohydrate complex of the end-piece, and that the end-piece acts as a carrier for the fourth component-carbohydrate complex.

These findings may also explain the past failures to purify complement by fractionation with ammonium sulfate because, if care is not taken to maintain a low temperature and a slightly acid pH, inactivation of complement readily occurs.

THE EFFECT OF SALT CONCENTRATION, TEMPERATURE AND DIALYSIS ON COMPLEMENT AND ITS COMPONENTS

Dialysis of whole serum against distilled water separates complement into a "globulin fraction" and an "albumin fraction."[31] The "globulin fraction" is termed "mid-piece." Mid-piece is unstable in 0.9 per cent saline.[60] In fact, it loses its activity in a few hours. However, it remains active and stable if suspended in distilled water[60, 61] or hypertonic salt solution.[62] It is inactivated by heating at 54° C. for 30 minutes,[63] and is non-dialyzable against water, hypotonic or hypertonic salt solutions.[64, 65]

The "albumin fraction" or "end-piece" is relatively stable on standing. At 2° C. its activity remains unimpaired for several days.[60, 66] It is also inactivated by heating at 54° C. for 30 minutes,[63] instead of at 56° C. for 30 minutes as had been previously reported. It is also non-dialyzable against water, hypotonic or hypertonic salt solutions.[64, 65]

The fourth component has a stability of the same order as the end-piece.[42] It is non-dialyzable,[65] but has a higher thermal inactivation, a temperature of 65° C. for 30 minutes being required to inactivate it completely.[63] In 10 per cent NaCl the thermostability of the fourth component is reduced to 61° C. for 30 minutes.

The third component is very unstable, and is the first to disappear in serum on standing at room temperature or on ice.[1, 65] It is non-dialyzable,[65] and is inactivated at a temperature of 62° C. for 30 minutes.[63] In 10 per cent NaCl the inactivation temperature is reduced to 60° C. for 30 minutes. Hypertonic salt solutions have a protective action on the removal of the third component from whole serum by yeast, zymin or the insoluble carbohydrate of yeast.[67]

THE ROLE OF CALCIUM AND THE EFFECT
OF LIPID SOLVENTS

Calcium

It has been generally assumed that serum calcium is associated with complement function, although differences of opinion exist as to the nature and action of the calcium. Some authors state that the removal of the diffusible calcium does not influence complementary activity;[5] but others claim that ionized calcium is necessary.[68]

The present authors investigated the role of calcium in complement constitution and function.[45] It was found that, whenever calcium was removed from an acid medium, a certain degree of acid inactivation took place; but the removal of calcium up to 9S per cent from a neutral or slightly alkaline medium had no effect on any component of complement. Calcium therefore plays a role of doubtful significance in complement function.

The results of these experiments are given in TABLE 4. It is noted that calcium dis-ionizing organic acids inactivate complement in a manner similar to the hydrogen-ion effect of HCl (see TABLE 2). However, their alkali salts, in amounts up to 5 per cent, had no effect on

TABLE 4‡

THE EFFECT OF CALCIUM DISIONIZING ORGANIC ACIDS AND THEIR ALKALI SALTS,
OF SODIUM HEXAMETAPHOSPHATE AND LEAD PHOSPHATE ON COMPLEMENT.

Complement treated with:	pH*	Per cent hemolysis†	Heated serum	NH_4OH serum	Zymin serum	End-piece	Mid-piece	Heated end-piece	Heated mid-piece
				Reactivations with:					
				Percentile restoration of complementary activity					
NH_4OH	7.2	0	90	0	100	100	10	85	0
Citric acid	4.9	0	25	0	80	100	10	15	0
Lactic acid	4.8	0	25	0	80	100	10	10	0
Tartaric acid	4.9	0	25	0	90	100	5	10	0
Oxalic acid	4.9	0	20	0	100	100	5	10	0
$Pb_3(PO_4)_2$	7.2	0	0	80	0	0	60	0	0
5% $Na_2(Na_4P_6O_6)$	6.8	100							
5% alkali citrates, oxalates and tartrates	7.2	100							

* Adjusted to 7.2 before titration.
† Titer of original serum taken as 100 per cent.
‡ This table is taken from Pillemer, L., & Ecker, E. E.   Jour. Immunol. 40: 101. 1941.

complement. It is also noted that sodium hexametaphosphate, which places the calcium so effectively in the anion complex that it is no longer available for serum analysis, likewise had no anti-complementary effect. Lea phosphate adsorbs 95 per cent of the serum calcium, as well as the mid-piece but there is no effect on the fourth component which supposedly contains calcium.

These results show that no definite relationship exists between calcium and the fourth component. In fact, the weight of evidence indicates that it is not involved in the immediate labile and reversible reactions of complement. It is the belief of the authors that calcium may be a structural part of the end-piece - fourth component complex, but not essential to its function, because, when acids are used to remove the calcium, the removal is accomplished at the expense of protein denaturation and the destruction of end-piece structure. The relationship of calcium to complement as observed by other investigators then becomes an index of the degree of complement denaturation.

Lipid Solvents

It is known that the treatment of serum with ether,[5] followed by the removal of the ether, inactivates complement. It was later reported that the factor destroyed by ether or chloroform was the fourth component.[5] As benzene inactivated a supposedly new fraction of complement, a hypothetical "fifth component" was postulated, but its presence was not confirmed.[9]

The present authors showed that the extraction in the cold of active dehydrated complement with absolute alcohol, anhydrous ether, or petroleum ether does not inactivate complement.[69] In fact, the extracted lipids were anti-complementary, and complement often showed an increase of activity after such extractions. As stated above, no evidence of a possible "fifth component" was found.

In addition,[7] it has been shown that treatment of fresh serum with ether, chloroform, cadmium chloride, benzene or benzine had no specific effect on any one component, but inactivated fresh complement by virtue of protein denaturation. While it is agreed that the phospholipids may play a role in complementary function, it has not as yet been possible to establish a definite relationship.

THE ACTIVE ANTI-COMPLEMENTARY PRINCIPLE IN FRESH YEAST

Although the inactivation of complement by yeast was described as early as 1900,[34] the nature of this inactivation remained obscure.

The present authors[3] isolated an insoluble fraction from fresh yeast which is composed of 94 per cent carbohydrate and is insoluble in hot water, organic solvents and cold alkali. The yield was about 2 per cent.

This insoluble carbohydrate fraction inactivated specifically the third component of complement in an amount of only one twenty-fifth of the required amount of fresh yeast. None of the soluble fractions from yeast inactivated the third component. Since the anti-complementary factor is undoubtedly an insoluble complex carbohydrate the inactivation of third component appears to be due to the adsorption of this relatively heat resistant component of blood serum.

EARLY ATTEMPTS AT THE PURIFICATION OF COMPLEMENT

Adsorption

As early as 1906 it was shown that complement is removed from serum by coagulated serum proteins.[71] Since that time many attempts have been made to adsorb complement on various adsorbing agents. Berkefeld filter, kaolin, alumina, carbon, insoluble calcium and magnesium compounds, barium sulfate, starch, inulin, agar, gelatin, and even bacteria have been used for this purpose.

While it has been suggested that the adsorbents remove only the mid-piece,[2] most investigators agree that no selective adsorption of any component occurs.[1, 71]

In view of the successes obtained in the purification of enzymes by adsorption and elution, controlled adsorption was undertaken in attempts to isolate or purify complement.[67] However, elution of the adsorbed complement and its components was found to be impossible, but certain observations were made in the course of these experiments and they are as follows: 1) Lead phosphate and titanium oxide remove the mid-piece of complement. 2) Kaolin and magnesium hydroxide are less specific in their action. 3) Aluminum hydroxide gel removes the total complement complex. 4) A correlation exists between mid-piece and third component and the amount of euglobulin phosphorus adsorbed.

Fractionation with Ammonium Sulfate

Although serious attempts have been made to separate complement from whole serum, the results have been too indefinite to allow the characterization of either complement or any of its components as functionally distinct entities. Also, past failures to purify complement can be attributed to lack of care in avoiding denaturation of the labile complement.

However, it was recently shown[72] that the addition of one volume of guinea pig serum to 14 volumes of 2.4 M ammonium sulfate results in the precipitation of about 35 per cent of the total serum proteins and that this precipitate contains, after the removal of the ammonium sulfate, about 90 per cent of the complementary activity originally present in the serum. Nevertheless, attempts to separate this material into the various components of complement were futile. It then became evident that, although the 1:15 dilution of the serum with ammonium sulfate solution sufficed to separate the globulins from serum without much loss of complementary activity, the same method was unsatisfactory for further purification of the complement components.

In FIGURE 1 is presented a fractional precipitation curve of the guinea pig serum globulins.* It is noted that there are four distinct salting-out plateaus. As to the complement component content of these fractions, the following were noted: (1) The material precipitating between 1.40 M and 1.56 M ammonium sulfate had a small amount of mid-piece activity, but was very unstable. (2) The proteins precipitating between 1.56 M and 2.2 M ammonium sulfate contained varying amounts of third com-
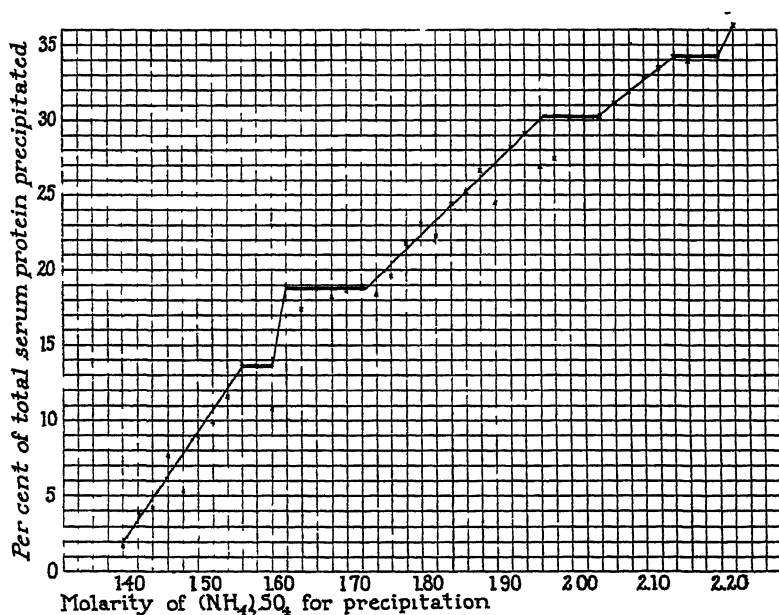

FIGURE 1. Fractional serum protein precipitation curve. Serum proteins precipitated from 1:15 serum at 2° C. by various concentrations of ammonium sulfate.

* Unpublished experiments performed by C. B. Jones and S. Seifter in collaboration with the authors.

ponent, fourth component and end-piece. However, the results were not clear-cut and characterization of any of these fractions proved to be impossible.

THE SEPARATION AND CHARACTERIZATION OF THE COMPONENTS OF COMPLEMENT

With the collaboration of Prof. E. J. Cohn and Dr. J. L. Oncley of Harvard University, the present authors have been able to separate three of the components of complement in a high degree of purity and have characterized these components physico-chemically and immunologically.[65] This was accomplished with exact physico-chemical manipulations.

To guide the work described below, electrophoretic diagrams were prepared of mid- and end-piece (made by the carbon dioxide method) as well as of zymin- and ammonia-treated sera.[73] The results indicate that the so-called "globulin fraction" or mid-piece contains at least four distinct proteins, two of which have electrophoretic mobilities faster than those originally present in whole serum; while the so-called "albumin fraction" or end-piece also contains at least four distinct proteins, one of which appears to carry nearly all of the γ-globulin originally present in whole serum. No significant difference was detected electrophoretically between normal serum and serum deprived of its fourth component by treatment with ammonia. Serum deprived of its third component by treatment with zymin showed a disturbance of the α-globulins, accompanied by a slight increase of the mobilities of the remaining serum proteins. It is evident, therefore, that the terms "mid-piece," "end-piece," "albumin fraction," and "globulin fraction" are unsatisfactory and misleading as applied here. Therefore, after discussions and agreement with Dr. M. Heidelberger of Columbia University, the components of complement have been designated as C'1, C'2, C'3 and C'4,[73] corresponding to the mid-piece, end-piece, third component, and fourth component respectively in the older terminology.

However, to avoid confusion, in this paper the older terminology is used in designating the components, but the suggested terminology will be followed in all subsequent publications from this laboratory.

Since the components involved in complement for hemolysis appear to be associated with serum proteins, it seemed important to isolate them in the highest degree of purity obtainable and to characterize them. This was successfully accomplished with three of the components of complement, *i.e.*, mid-piece, end-piece, and fourth component. The separation was achieved under rigidly controlled conditions by fractional precipitation with ammonium sulfate, accompanied by various extrac-

tions and dialysis procedures. The method is published in detail elsewhere.[65]

Mid-piece was found to be a euglobulin with an apparent iso-electric point of 5.2; with an electrophoretic mobility of 2.9 x 10^{-5} in phosphate buffer of ionic strength 0.2 at pH 7.7; and with a sedimentation constant of 6.4 x 10^{-13} in KCl of ionic strength 0.2. Its activity was destroyed by heating at 50° C. for 30 minutes, by hydrogen ion concentrations alkaline to its iso-electric point, and also in dilute solutions. It exhibited a definite inhibitory effect in protein concentrations exceeding 0.02 per cent, while full activity was observed at protein concentrations between 0.002 and 0.02 per cent. It comprised 0.60 per cent of the total serum proteins.

The end-piece and fourth component were present in the same serum fraction. The final purified protein was a muco-euglobulin, 98 per cent of which had an electrophoretic mobility of 4.2 x 10^{-5}. The final yield was 0.18 per cent of the total serum proteins. Its apparent iso-electric point was about 6.3-6.4. It contained 10.3 per cent carbohydrate, and had a specific optical rotation of $-192.5°$. Immunologically, this serum fraction appeared to play a dual role. Treatment at 50° C. for 30 minutes destroyed all of the end-piece activity, while the fourth component was totally destroyed only by heating at 66° C. for 30 minutes. The fraction therefore performed two immunological functions, one by virtue of a heat-labile constituent, and the other by virtue of a relatively heat-stable constitutent.

The properties of these purified fractions are contrasted in TABLE 5. It is noted that the apparent iso-electric point, that is, the pH of water triturated with the precipitated euglobulins associated with end-piece and fourth component, actually was close to 6.3, while that of the mid-piece was 5.2. The fraction with the higher mobility and precipitated by the higher concentration of salt thus had the more nearly neutral iso-electric point. A more detailed study of this unexpected result will be undertaken in order to examine the interactions of these components of complement with one another, and with the other components of serum.

Third component was found in small quantities in nearly every fraction of serum. Attempts at the purification of this component by specific adsorption with the insoluble carbohydrate from fresh yeast[15] and subsequent elution are now in progress.

The three purified complement components (mid-piece, end-piece and fourth component) comprise about 0.8 per cent of the total serum proteins. Recently,[74] employing an entirely different technique (fixation) it has been found that one cc. of guinea pig serum contains from 0.15 to

0.2 mg. of complement protein.   However, in a personal communication to the authors, Heidelberger now reports that he finds 0.4 to 0.7 mg. of complement protein to be a safer approximation.   This is in good agreement with the values found by fractional precipitation.   The variability of the amount of complement fixed by an antigen-antibody complex may be due to the relative amounts of each component fixed.   Work now in progress in this laboratory points to such a possibility.

TABLE 5*

CHARACTERIZATION OF THE MID-PIECE
AND THE END-PIECE AND THE FOURTH COMPONENT OF COMPLEMENT

	Euglobulin (mid-piece)	Muco-euglobulin (end-piece and fourth component)
Electrophoretic mobility x 10^5 pH 7.7; ionic strength 0.20	2.9	4.2
$S = \frac{1\%}{20^\circ},_w$ x 10^{13} In potassium chloride of ionic strength 0.20	6.4	
Per cent protein nitrogen	16.3	14.2
Per cent total serum protein	0.72	0.17
Per cent carbohydrate	2.7	10.3
Per cent phosphorus	<0.1	<0.1
[α] 25° C.	−28.7°	−192.5°
Apparent iso-electric point	5.2–5.4	6.3–6.4
Percent original complementary activity	100	85
Heat stability	Destroyed at 50° C. for 30 minutes.	End-piece destroyed at 50° C. for 30 minutes; fourth component at 66° C. for 30 minutes.

* This table is taken from Pillemer, L., Ecker, E. E., Oncley, J. L., & Cohn, E. J.   Jour. Exp. Med 74: 297.   1941.

CONCLUSIONS

It is now established that three of the components of complement are distinct chemical entities.   Therefore, past contentions that surface tension or collodial states alone can explain complementary activity are no longer tenable.   In fact, the complementary activity of serum may

now be viewed as the result of interactions between chemical entities, *and the destruction or removal of any of these substances alters the activity of the complement complex.*

Mention should be made that in the treatment of bacterial diseases, actively or passively, it has been assumed that complement is always present in abundance. Although it is now known that three of the complement substances comprise only about 0.8 per cent of the total serum proteins, it is evident that this is not a safe assumption, in that these factors, in infections, may be depleted faster than they are produced. They may therefore be of greater diagnostic value, and may even prove to possess prognostic and therapeutic significance.

REFERENCES

[1] Osborn, T. W. B. "Complement or Alexin." Oxford Univer. Press. London. 1937; Zinsser, H., Enders, J. F., & Fothergill, L. D. "Immunity: Principles and Applications in Medicine and Public Health." Macmillan. New York. 5th ed. 1939.

[2] Brocq-Rousseu, D., & Roussel, G. "Le Sérum Normal." Masson & Co. Paris. 1939.

[3] Pfeiffer, R., & Issaeff, R. Ztschr. Hyg. u. Infektionskrankh. 17: 355. 1894.

[4] Ledingham, J., & Dean, H. R. Jour. Hyg. 12: 152. 1912.

[5] Ward, H. K., & Enders, J. F. Jour. Exp. Med. 57: 527. 1933.

[6] Dean, H. R. Proc. Roy. Soc. London. 84B: 416. 1912.

[7] Dean, H. R. Jour. Path. & Bact. 21: 193. 1917.

[8] Bordet, J., & Gay, F. P. Ann. Inst. Pasteur. 20: 467. 1906.

[9] Nuttall, G., Ztschr. Hyg. u. Infektionskrankh. 4: 353. 1888.

[10] Douglas, S. R., & Smith, W. Brit. Jour. Exp. Path. 11: 96. 1930.

[11] Magheru, G., & Magheru, A. Compt. rend. Soc. Biol. 101: 204, 206. 1929.

[12] Fuchs, H. J. Ztschr. Immunitätsforsch. 59: 424. 1928; 61: 342. 1929; 62: 107, 117. 1929; 67: 266, 272. 1930.

[13] Wadsworth, A., Maltaner, F., & Maltaner, E. Jour. Immunol. 33: 297. 1937.

[14] Bancroft, F. W., Quick, A. J., & Stanley-Brown, M. Proc. Soc. Exp. Biol. & Med. 31: 791. 1934.

[15] Quick, A. J. Jour. Immunol. 29: 87. 1935.

[16] Ecker, E. E., & Pillemer, L. Jour. Immunol. 40: 73. 1941.

[17] Bruschettini, G. Pathologica. 26: 319. 1934; Ohtsuka, S., Teras, H., & Sugiu, K. Okayama-Igakkai-Zasshi. 44: 2462. 1932; Tomasino, A. Cervello. 13: 1. 1934.

[18] Kraus, F., & Fuchs, H. J. Ztschr. ges. exp. Med. 64: 583. 1929; 68: 245. 1929.

[19] von Liebermann, L. Deut. med. Wchnschr. 47: 1283. 1921.

[20] Wassermann, A. Ztschr. Hyg. u. Infektionskrankh. 37: 173. 1901.

[21] Dick, G. F. Jour. Infect. Dis. 12: 111. 1913.

[22] Hill, A., & Parker, G. Jour. Path. & Bact. 28: 1. 1925.

[23] Ohtsuka, S., Teras, H., & Sugiu, K. Okayama-Igakkai-Zasshi. 44: 2462. 1932.

[24] Eagle, H., & Brewer, G. Jour. Gen. Physiol. 12: 845. 1929.

[25] Wright, H. D., & MacCallum, P. Jour. Path. & Bact. 25: 316. 1922.

[26] Gordon, J., & Thompson, F. C. Brit. Jour. Exp. Path. 14: 33, 277. 1933.

[27] Jacoby, M., & Schütze, A. Klin. Wchnschr. 46: 2139. 1909.

[28] Brooks, S. C. Jour. Med. Res. 33: 345. 1918.

[29] Michaelis, L., & Skwirsky, P. Ztschr. Immunitätsforsch. 7: 497. 1910.

[30] Craigie, J. Brit. Jour. Exp. Path. 12: 75. 1931.

[31] von Buchner, H. Arch. Hyg. 17: 179. 1893.

[32] Liefmann, H. Münch. med. Wchnschr. 56: 2097. 1909.

[33] Sachs, H., & Altmann, K. Cited from "Handbuch der Technik und Methodik der Immunitätsforschung." 2: 969. 1909.

[34] von Dungern, E. Münch. med. Wchnschr. 47: 677. 1900.

[35] Gordon, J., Whitehead, H. R., & Wormall, A. Biochem. Jour. 20: 1028, 1036, 1044. 1926

[36] Pillemer, L., Seifter, S., & Ecker, E. E. Proc. Soc. Exp. Biol. & Med. 45: 130. 1940.

[37] Müller, L. Cent. Bakt. (Orig.) 57: 577. 1910.

[38] Brooks, S. C.   Jour. Med. Res. **41**: 399.   1920.

[39] Eagle, H.   Jour. Gen. Physiol. **12**: 821, 825.   1929.

[40] Osborn, T. W. B.   Biochem. Jour. **25**: 2136.   1931; **27**: 1425.   1933.

[41] Koopman, J., & Falker, I. D.   Jour. Lab. & Clin. Med. **21**: 312.   1935; Ecker, E. E., Pillemer, L., Wertheimer, D., & Gradis, H.   Jour. Immunol. **34**: 19.   1938.

[42] Pillemer, L., Seifter, J., & Ecker, E. E.   Jour. Immunol. **40**: 89.   1941.

[43] Pillemer, L., & Ecker, E. E.   Jour. Immunol. **40**: 101.   1941.

[44] Eagle, H.   "The Laboratory Diagnosis of Syphilis."   C. V. Mosby Co.   St. Louis.   1937.

[45] Pillemer, L., & Ecker, E. E.   Jour. Biol. Chem. **137**: 139.   1941.

[46] Tokunaga, H.   Cent. Bakt. (Abt. I).   **107**: 283.   1928.

[47] Parsons, E. I.   Jour. Immunol. **12**: 47.   1926.

[48] Browning, C. H., & Mackie, T. J.   Ztschr. Immunitätsforsch. **21**: 422.   1914; Browning, C. H.   "Immunochemical Studies."   Constable Co.   London. p. 180.   1925.

[49] Löw, W.   Wien. klin. Wchnschr. **35**: 12.   1922.

[50] Marsh, F.   Nature. **137**: 618.   1936.

[51] Hargan, E. S.   Nature. **137**: 872.   1936.

[52] Harde, E., & Thomson, A. E.   Compt. rend. Acad. **200**: 1425.   1935.

[53] Torrance, C. C.   Ann. Rep. Div. Labs. & Res. N. Y. State Dept. Health.   Albany. p. 29   1939.

[54] Ecker, E. E., Pillemer, L., Griffitts, J. J., & Schwartz, W. P.   Jour. Amer. Med. Assn. **112**: 1449.   1939.

[55] Chu, F., & Chow, B. F.   Proc. Soc. Exp. Biol. & Med. **38**: 679.   1938.

[56] Capelli, F.   Pathologica. **28**: 410.   1936.

[57] Ecker, E. E., Pillemer, L., Martiensen, E. W., & Wertheimer, D.   Jour. Biol. Chem. **123**: 351.   1938.

[58] Toda, T., & Mitsuse, B.   Ztschr. Immunitätsforsch. **78**: 62.   1933.

[59] Tokano, Y.   Ztschr. Immunitätsforsch. **87**: 29, 72.   1936.

[60] Brand, H.   Klin. Wchnschr. **44**: 1075.   1907.

[61] Pillemer, L., & Ecker, E. E.   unpublished.

[62] Pillemer, L., & Ecker, E. E.   unpublished.

[63] Ecker, E. E., Pillemer, L., & Kuehn, A. O.   Proc. Soc. Exp. Biol. & Med. **45**: 115.   1940.

[64] Jones, C. B., & Ecker, E. E.   Proc. Soc. Exp. Biol. & Med. **44**: 264.   1940.

[65] Pillemer, L., Ecker, E. E., Oncley, J. L., & Cohn, E. J.   Jour. Exp. Med. **74**: 297.   1941.

[66] Pillemer, L., & Ecker, E. E.   unpublished.

[67] Ecker, E. E., Jones, C. B., & Kuehn, A. O.   Jour. Immunol. **40**: 81.   1941.

[68] Ottolenghi, D., & Mori, M.   Cent. Bakt. **38**: 338, 468.   1905.

[69] Ecker, E. E., Pillemer, L., & Grabill, F. J.   Proc. Soc. Exp. Biol. & Med. **38**: 318.   1938.

[70] Landsteiner, K., & Stankovic, R.   Cent. Bakt. **41**: 108.   1906.

[71] Strong, P. S., & Culbertson, J. T.   Jour. Hyg. **34**: 522.   1934.

[72] Ecker, E. E., Pillemer, L., Jones, C. B., & Seifter, S.   Jour. Biol. Chem. **135**: 347.   1940.

[73] Pillemer, L., & Ecker, E. E.   Science. **94**: 437.   1941.

[74] Heidelberger, M.   Science. **92**: 584.   1940.

THE QUANTITATIVE RELATIONSHIP BETWEEN ANTIGEN AND ANTIBODY IN THE PRECIPITIN REACTION

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Heidelberger and his co-workers, by the application of quantitative methods to the study of the precipitin reaction, have shown that for many systems the relationship between antibody and antigen in the precipitate formed when antigen is added to an excess of antibody can be expressed by the equation,

$$y = 2Rx - \frac{R^2 x^2}{A}$$

where y = mg. antibody precipitated.

  x = mg. antigen added.

  A = Total antibody present.

  R = Ratio $\dfrac{\text{Antibody}}{\text{Antigen}}$ at the equivalence point.

Although good agreement is obtained between the values calculated from this equation and those found experimentally for many systems which have been carefully studied,[1] in other systems certain changes must be made in the equation in order to obtain agreement.[1]

In 1935 it was shown by Heidelberger and Kendall[1a] that this equation could be derived by an application of the law of mass action if certain assumptions were made. The authors were aware that the assumptions upon which this derivation was based were over simplified. Therefore, although the derived relationship explained the two most striking *quantitative* aspects of the precipitin reaction, namely, the changing ratio between the two components in the precipitate and the lack of a pronounced volume effect, it is necessary to reexamine the theoretical basis upon which it rests.

The following assumptions form the basis of a modified theory. Some of the assumptions rest upon independent evidence so substantial that they must be accepted, and others are logical conclusions drawn from available information concerning the properties of antigens and antibodies.[2] The assumptions are:

1. Antibodies are modified serum proteins.
2. The reaction between antigen and antibody is between specific groups or areas upon the surface of the molecules.

3. Both antigen and antibody may be multivalent with respect to each other, *i.e.*, each molecule may possess more than one reactive group or area. The reactive groups upon a given molecule may all have the same specificity or they may be different. The maximum number of molecules of antibody bound by one antigen molecule may be determined by the number of reactive groups upon the antigen or it may be limited by steric factors. There is a limit to the number of antibody molecules that can be arranged about an antigen molecule of finite size.

4. Within the limits imposed by steric factors the reactivity of any specific group upon the antigen or antibody molecule is independent of the state of combination of other specific groups upon the same molecule. The reactivity of an antigen or antibody group upon the surface of a precipitate is the same as the reactivity of the same group on the surface of a molecule in solution.

5. An equilibrium is established between the free and combined antigen and antibody groups in the system. It is evident that the system under consideration is not homogeneous. It is concerned with the reaction of groups upon larger surfaces and the equilibria established will be between reactive groups upon the surface of precipitates and the molecules in solution.

Inasmuch as both antigens and antibodies are complex protein molecules which may vary widely in their properties a satisfactory theory must allow for these variations.

Thus, antigens may vary in molecular weight and in the number and kind of reactive groups upon their surfaces. In some cases all of the reactive groups may react as if they had the same specificity, whereas in others part of the groups may react with separate antibodies. The reactive groups may be arranged upon the antigen in such a way that they react independently of each other. They may also be arranged in such a way that the reaction of one group prevents the reaction of other groups.

The antibody may also show the same type of variations. The antiserum produced by immunizing an animal with any antigen may contain a mixture of antibody molecules which differ in the number and kind of reactive groups and in the firmness with which they combine with the antigen.

Because of the complex possibilities of antigen-antibody systems the discussion of the theoretical consequences of the assumptions made will be divided into a number of sections. The theory will be developed first for the simpler cases and then extended to the more complicated systems.

In the first case to be considered, it is assumed that the reaction is irreversible and that all of the reactive groups upon the antigen and all the antibody molecules in the antiserum are alike.

If the valence of antibody is 1, in the presence of an excess of antibody all of the reactive groups upon the antigen will be combined with whole antibody molecules. The relationship between A, the number of antibody molecules combined, and B, the number of antigen molecules added, would be

$$A = NB \text{ where } N = \text{valence of antigen,}$$

or, *Mg. Antibody pptd. = $rN \times$ mg. antigen added,*

where r is the ratio between the molecular weights of antibody and antigen.

If the valence of antibody is 2, in the presence of an excess of antibody all of the antigen groups will be in combination, but some of the antibody molecules will have both groups free, some will have one group combined, and some will have both groups combined. If there is a perfectly random distribution of the available antigen groups between the reactive groups upon the antibody, the most probable number of molecules of antibody combined in the different ways can be computed.

Let $\quad A$ = Number of molecules of antibody in the system.

$\quad B$ = Number of molecules of antigen added.

$\quad N$ = Valence of antigen.

$\quad NB$ = Number of antigen groups added, and also

$\quad\quad\quad$ = Number of antibody groups combined.

$\dfrac{NB}{2A}$ = Fraction of antibody groups combined and also the probability that any particular antibody group is combined.

$\dfrac{2A - NB}{2A}$ = Probability that any particular antibody group is free.

$\left(\dfrac{2A - NB}{2A}\right)^2$ = Probability that any antibody molecule has both groups free.

$\left(\dfrac{NB}{2A}\right)^2$ = Probability that any antibody molecule has both groups combined.

$2\left(\dfrac{2A - NB}{2A}\right)\left(\dfrac{NB}{2A}\right)$ = Probability that any antibody molecule has one group free and one combined.

The number of uncombined antibody molecules would then be

$$\frac{2A - NB}{2A}\bigg)^{2} A = A - NB + \frac{\overline{NB^2}}{4A}.$$

The number of singly combined antibody molecules would be

$$2\left(\frac{2A - NB}{2A}\right)\left(\frac{NB}{2A}\right)A = NB - \frac{\overline{NB^2}}{2A}.$$

The number of doubly combined antibody molecules would be

$$\left(\frac{NB}{2A}\right)^{2} A = \frac{\overline{NB^2}}{4A}.$$

The total number of combined antibody molecules would be

$$A \text{ combined} = NB - \frac{\overline{NB^2}}{4A}.$$

If $R = \dfrac{\text{mol. wt. antibody}}{\text{valence antibody}} \times \dfrac{\text{mol. wt. antigen}}{\text{valence antigen}}$,

this formula reduces to

$$\text{Mg. antibody pptd.} = 2Rx - \left(\frac{R^2 x^2}{A}\right),$$

where x = mg. antigen added.

If the valence of antibody is M, then

$$\left(\frac{MA - NB}{MA}\right)^{M} A = \text{number of uncombined antibody molecules.}$$

$$A - \left(\frac{MA - NB}{MA}\right)^{M} A = \text{number of combined antibody molecules.}$$

The number of antibody molecules having 0, 1, 2, and 3 ....M groups combined is given by the successive terms of the binomial expansion:

$$\left(\frac{MA - NB}{MA} + \frac{NB}{MA}\right)^{M}.$$

The second case to be considered is one in which the reaction is reversible and an equilibrium is set up between free and combined reactive groups. The system must be treated as a heterogeneous system in which groups upon molecules in solution are in equilibrium with free and combined reactive groups upon surfaces. As before all of the reactive groups upon the antigen are considered to be alike.

If the valence of antibody is 1, equilibrium would be established between the number of free and combined groups upon the antigen, which is considered to form the surface phase, and the concentration of antibody in solution.

If A = Total number of antibody molecules in the system.

B = Number of antigen molecules added.

N = Valence of antigen.

x = Number of antigen groups in combination, and also

= Number of antibody groups in combination.

$(NB - x)$ = Number of free antigen groups.

$(A - x)$ = Number of free antibody molecules.

at equilibrium,

$$(NB - x) \frac{(A - x)}{V} = kx.$$

This equation reduces to a form which is the same as that derived by Ghosh[3] with the exception that it includes a volume term.

If the valence of antibody is 2, the groups upon either the antigen or antibody may be considered to constitute the surface phase which is in equilibrium with the reactive groups upon the molecules in solution. The reaction may be treated the same as in the case of the irreversible reaction with the exception that the number of antigen groups in combination will not be NB, the total number of antigen groups added, but will be some smaller number x determined by the magnitude of the dissociation constant. If there is a random distribution of x combined groups between the antigen and antibody molecules in the system,

$$\left(\frac{2A - x}{2A}\right)^2 A = \text{Number of uncombined antibody molecules.}$$

$$2\left(\frac{2A - x}{2A}\right)\left(\frac{x}{2A}\right)A = \text{Number of antibody molecules with one group combined}$$

$$\left(\frac{x}{2A}\right)^2 A = \text{Number of antibody molecules with both groups combined.}$$

$$(NB - x) = \text{Number of free antigen groups.}$$

If equilibrium is established in the system as a whole it will also be established in each step of the reaction. The first step will be the reaction of *one group* of an antibody molecule in solution with a reactive group upon the antigen.

$$A + b \rightleftharpoons Ab$$
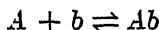
The rate of combination will be proportional to the *concentration* of antibody groups in solution times the *number* of uncombined antigen groups. The rate of dissociation will be proportional to the number of antibody molecules with one group combined. At equilibrium

$$(NB - x)\left(\frac{2A - x}{2A}\right)^2\left(\frac{2A}{V}\right) = k\left(\frac{2A - x}{2A}\right)\left(\frac{x}{2A}\right)(2A).$$

This simplifies to the expression,

$$(NB - x)(2A - x) = kVx.$$

If the antibody groups are considered to constitute the surface phase in equilibrium with the antigen groups on the antigen molecules in solution, the equation involved becomes

$$(2A - x)\left(\frac{NB - x}{NB}\right)^N\left(\frac{NB}{V}\right) = k\left(\frac{NB - x}{NB}\right)^{N-1}\left(\frac{x}{NB}\right)NB$$

which also simplifies to the same form.

The amount of antibody precipitated will be $x - \frac{x^2}{4A}$ instead of $NB - \frac{\overline{NB^2}}{4A}$, x being defined by the equation given above. If the dissociation constant is small the difference between these two values becomes experimentally significant only in the equivalence zone where the excess of antibody is small. Except in this region the reaction may be satisfactorily expressed by the formula,

$$\text{Mg. antibody pptd.} = 2Rx - \frac{R^2x^2}{A}$$

where x is the mgs. of antigen added.

In these cases all of the reactive groups upon the antigen were assumed to be alike and the reactivity of one group was not affected by the reaction of the other groups on the same molecule. The necessity of these assumptions can be tested.

Let it be assumed that antigen has four groups that react with the same *bi-valent* antibody to form compounds that have different dissociation constants.

If A = Number of antibody molecules present.

B = Number of antigen molecules present.

x, y, z, and u are the numbers of the different groups that have reacted.

At equilibrium,

$$(2A - x - y - z - u)(B - x) = k_1Vx$$
$$(2A - x - y - z - u)(B - y) = k_2Vy$$
$$(2A - x - y - z - u)(B - z) = k_3Vz$$
$$(2A - x - y - z - u)(B - u) = k_4Vu$$

If values are assigned to the k's, relative values can be computed for the other terms in these equations. The amount of antibody combined will be,

$$(x + y + z + u) - \frac{(x + y + z + y)^2}{4A}$$

As shown in TABLE 1, the relative amounts of antibody combined by antigen which has 4 groups reacting with antibody with dissociation constants 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2}, are compared with the amount

precipitated by antigen in which all valences are alike and have a dissociation constant of 10^{-5}.

TABLE 1

Antigen added	Antibody combined	
	Mixed antigen	Simple antigen
0.1	0.1900	0.1900
0.2	0.3600	0.3600
0.3	0.5100	0.5100
0.616	0.8518	0.8526
0.764	0.9434	0.9443
0.802	0.9600	0.9608

From the data summarized in TABLE 1, it can be seen that it would be impossible to distinguish experimentally between the two systems.

Another case has been considered in which it is assumed that all of the groups upon the antigen are alike, but that the reaction of each group lowers the tendency for the remaining groups to react with antibody. The reaction is considered to take place in steps and the dissociation constant is assumed to increase by a factor of 10 for each step. If x, y, z, and u represent the number of molecules of antigen with 1, 2, 3 and 4 reactive groups combined, the equilibrium equations would be:

$$(2A - x - 2y - 3z - 4u) \cdot 4(B - x - y - z - u) = k_1 Vx$$
$$(2A - x - 2y - 3z - 4u) \cdot 3(x) = 2k_2 Vy$$
$$(2A - x - 2y - 3z - 4u) \cdot 2(y) = 3k_3 Vz$$
$$(2A - x - 2y - 3z - 4u) \cdot (z) = 4k_4 Vu$$

Relative values for A, B, x, y, z and u can be computed from these equations and the amount of bivalent antibody bound by different amounts of antigen can be calculated.

In TABLE 2 is depicted a comparison between the relative amounts of

TABLE 2

Antigen added	Antibody combined	
	Variable k $k = 10^{-5} - 10^{-2}$	Fixed k $k = 10^{-5}$
0.231	0.4090	0.4092
0.335	0.5566	0.5572
0.405	0.6456	0.6458
0.536	0.7518	0.7536
0.670	0.8910	0.8934
0.850	0.9738	0.9770

antibody combined with antigen in this system and the amount bound by antigen with a fixed dissociation constant.

From the results set forth in TABLE 2, it would be impossible to distinguish analytically between the two systems in the region of antibody excess.

It has been shown that it makes little difference quantitatively whether or not all of the groups upon the antigen react alike. What would be the effect if the two reactive groups upon the antibody molecule were different in their reactivity? Assume that the antibody molecule has two reactive groups A_1, and A_2, that react with the same groups upon the antigen molecules, and that the dissociation constants k_1, and k_2 are of different magnitudes.

If A = Number of antibody molecules.

B = Number of antigen molecules.

x = Number of A_1 groups combined.

y = Number of A_2 groups combined.

at equilibrium,

$$(A - x)\,(NB - x - y) = k_1 Vx.$$
$$(A - y)\,(NB - x - y) = k_2 Vy.$$

Mols. antibody combined $= x + y - \dfrac{xy}{A}.$

Assuming values for A, k_1, and k_2 the relative values for NB, x and y can be calculated. FIGURE 1 compares the curve obtained for a system where $A = 1.0$, $k_1 = 10^{-5}$, $k_2 = 10^{-4}$ with the curve obtained if both of the antibody groups were alike. One system could be distinguished from the other experimentally.

If the antibody has two reactive groups reacting with different kinds of groups upon the antigen molecule,

N_1 = Number of the first kind of group on the antigen molecule.

N_2 = Number of the second kind of group on the antigen molecule.

x = Number of A_1 groups combined.

y = Number of A_2 groups combined.

$$(A - x)\,(N_1B - x) = k_1 Vx.$$
$$(A - y)\,(N_2B - y) = k_2 Vy.$$

If k_1 and k_2 are small, x approximates N_1B and y approximates N_2B, except near the equivalence point.

$$\text{Mols. antibody combined} = (N_1 + N_2)B - \frac{N_1 N_2 B^2}{A}.$$

If $N_1 = N_2$, the equation becomes identical with the one obtained for antibody with both groups alike. For all other values for N_1 and N_2 the amount of antibody combined will be greater than for the simple system.
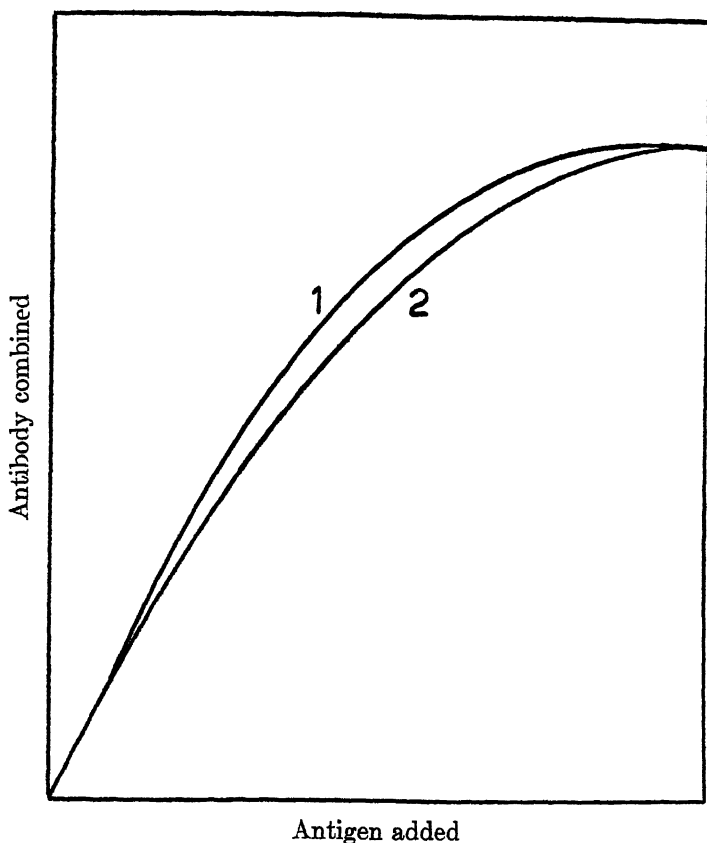
FIGURE 1. Reaction of non-homogeneous antibody. The curves show the effect of a difference in the reactivity of the two reactive groups of bi-valent antibody.

1. $k_1 = 0.1k_2$;
2. $k_1 = k_2$.

FIGURE 2 shows the types of curves obtained when $N_1 = N_2$ and $N_1 = 5N_2$.

The antiserum formed by immunizing a rabbit with an antigen may contain a mixture of antibodies reacting with different specific groups upon the antigen molecule. A great number of variations are possible in such a system.

1. If the number and position of the reactive groups upon the antigen permit the independent reaction of each group the amount of antibody precipitated from a mixed antiserum will be the sum of the amounts calculated for each individual system. If A_1, A_2, etc., represent the amount of the different antibodies present and N_1, N_2, etc., represent the

number of the different reactive groups upon an antigen molecule, the amount of monovalent antibody combined will be

$$N_1B + N_2B + etc.,\ (\text{if } k_1, k_2, etc., \text{ are small})$$

as long as an excess of all kinds of antibody is present. If the different antibodies are present in varying amounts so that some of them are completely precipitated while others are present in excess this expression becomes

$$\text{Antibody combined} = A_1 + A_2 + N_3B + \ldots etc.$$

If antibody combined is plotted against antigen added a graph made up of several straight line facets would result.

If the antibody is bi-valent the amount combined will be

$$N_1B - \frac{\overline{N_1B}^2}{4A_1} + N_2B - \frac{\overline{N_2B}^2}{4A_2} + etc,$$

Antibody combined

Antigen added

FIGURE 2. Reaction of non-homogeneous antibody. The antibody molecule is assumed to have two groups that react independently with two different kinds of groups upon the antigen. The curves show the effect of a difference in the relative numbers of the antigen groups.

1. $N_1 = 5N_2$;
2. $N_1 = N_2$.

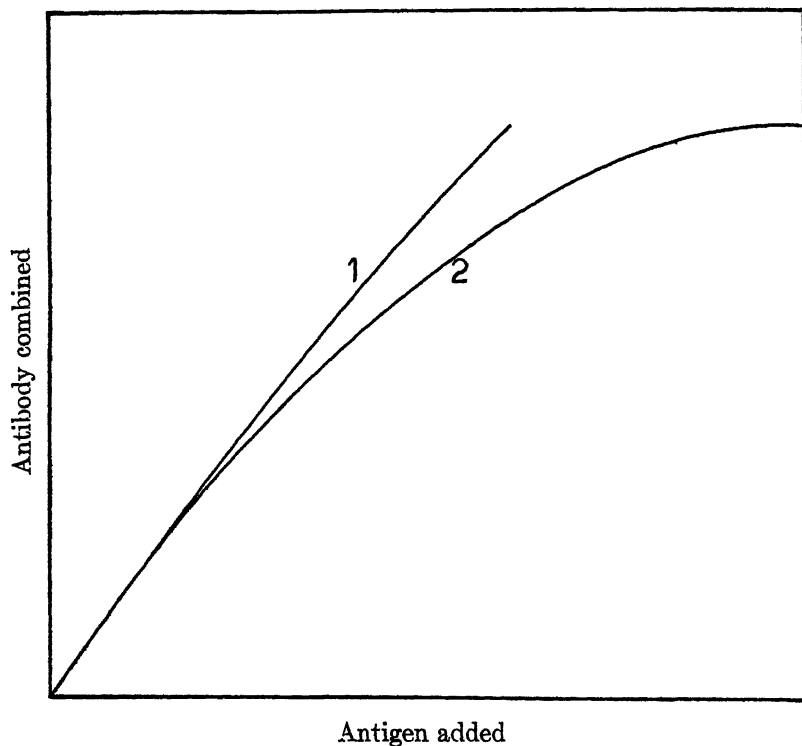as long as an excess of all kinds of antibody is present. If one of them is exhausted while an excess of the others is present the expression becomes,

$$A_1 + N_2B - \frac{\overline{N_2B}^2}{4A_2} + \text{etc.}$$

2. If the number or position of the reactive groups upon the antigen do not permit the independent reaction of each group many different cases are possible. The only case to be considered in detail is one in which there are two kinds of groups upon the antigen and the number and position of the groups prevent independent reaction of the two kinds of antibody. There will be a competition between the two kinds of antibody for the available groups. If $k_1 = k_2$ the system will react like a system in which all of the antibody is alike. If $k_1 \neq k_2$ the following relationships should hold for bi-valent antibody.

Let $N_1 = N_2 = N$ the number of available groups on the antigen.

$\quad A_1 =$ Mols. of antibody 1 present.

$\quad A_2 =$ Mols. of antibody 2 present.

$\quad x =$ Number of groups of antibody 1 in combination.

$\quad y =$ Number of groups of antibody 2 in combination.

At equilibrium,

$$(2A_1 - x)(NB - x - y) = k_1Vx.$$
$$(2A_2 - y)(NB - x - y) = k_2Vy.$$
$$y = \frac{2k'A_2x}{2A_1 - (1 - k')x} \text{ where } k' = \frac{k_1}{k_2}$$
$$\text{Antibody combined} = x - \frac{x_2}{4A_1} + y - \frac{y^2}{4A_2}$$

The values obtained for systems in which

$\quad A_1 = A_2, k_1 = 10^{-6}$ and $k_2 = 10^{-4}$ and

$\quad 3A_1 = A_2, k_1 = 10^{-6}$ and $k_2 = 10^{-4}$ are shown in FIGURE 3.

The theory presented here does not predict the way in which all antigen-antibody systems will react. No single equation could be adequate for all possible variations in such a system. Its value lies in the fact that it permits logical deductions to be made concerning the chemical nature of the antigen and antibody molecules from a quantitative study of their interaction.

The data obtained by Heidelberger and Kendall[1c] for the reaction of crystalline egg albumin with the antisera obtained after different periods of immunization of the same rabbit are analysed in the following discussion.

The theory predicts that the maximum ratio of antibody and antigen combined should be

$$R_{\text{max}} = \frac{\text{Molecular weight of antibody}}{\text{Molecular weight of antigen}} \times \text{valence of antigen.}$$
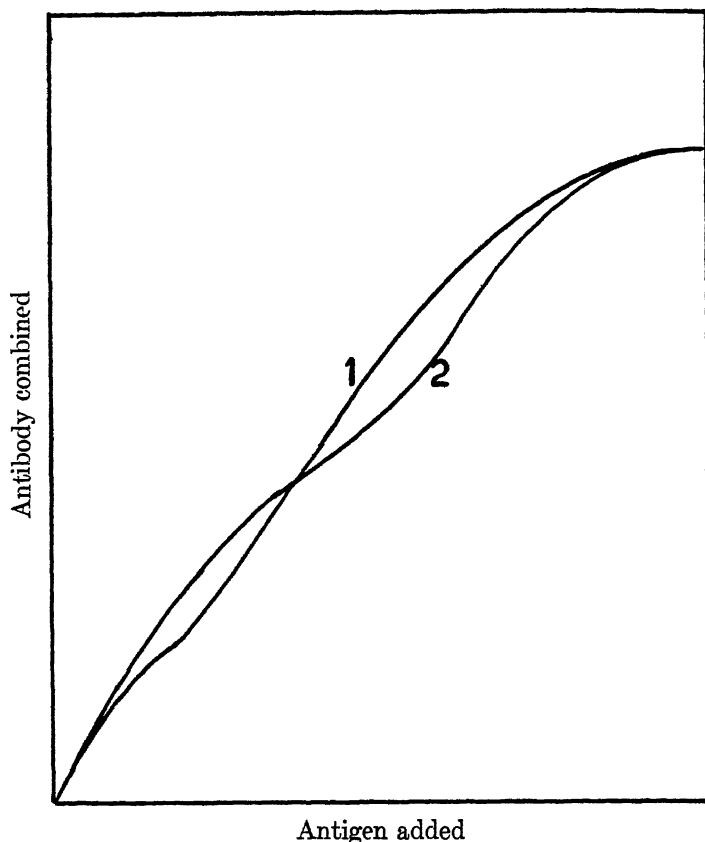
FIGURE 3.   Reaction of non-homogeneous antibody.   The antiserum is considered to contain two species of antibody molecules reacting with the same antigen groups.

$$1. \ 3.A_1 = A_2, \quad A_1 = 10^{-6}, \quad k_2 = 10^{-1};$$
$$2. \ .A_1 = .A_2, \quad k_1 = 10^{-6}, \quad k_2 = 10^{-4}.$$

Since the valence of antigen is an integral number this relationship predicts that the maximum ratio must be one of a limited number of values. Assuming molecular weights of 160,000 and 40,000 for the antibody and E. A., the possible ratios would be some multiple of 4.   The change in ratio as the amount of added antigen is increased can be due either to dissociation, which would leave increasing numbers of antigen groups uncombined with antibody as the concentration of free antibody in the supernatent diminished, or to polyvalency of the antibody molecules. If the valence of antibody is one the change in ratio must be due to dissociation and the reaction should follow the equation,

$$(A - x) \ (rB - x) = kVx,$$

where A = mgs. total antibody in the system.

B = mgs. antigen added.

r = maximum ratio.

x = mgs. antibody combined.

Since a high dissociation constant would make experimental determination of the value of A impossible this equation contains 3 constants, A, r and k, which must be evaluated from the experimental data. This has been done for this system. TABLE 3 shows that values can be found

TABLE 3

ANTI-EGG ALBUMIN RABBIT SERUM NUMBER 387

$(A-x)\,(rB-x)=kvx$								
1st Course $A=1.00$ $r=24$ $k=.23$ $v=2$ ml.			2nd. Course $A=1.3$ $r=24$ $k=.135$ $v=2$ ml.			3rd. Course $A=1.6$ $r=24$ $k=.08$ $v=2$ ml.		
Ea.	Antibody precipitated		Ea.	Antibody precipitated		Ea.	Antibody precipitated	
mg. N	Found *mg.* N	Calculated *mg.* N	*mg.* N	Found *mg.* N	Calculated *mg.* N	*mg.* N	Found *mg.* N	Calculated *mg.* N
.009	0.15	0.14	.015	0.29	0.32	.03	0.64	0.63
.015	.22	.23	.05	0.79	0.79	.049	0.96	0.94
.025	.35	.35	.088	1.06	1.04	.079	1.24	1.27
.040	.49	.50	.098	1.08	1.10	.082	1.29	1.30
.050	.58	.58	.118	1.10	1.12	.088	1.33	1.33
.065	.68	.67	.127	1.15	1.14	.098	1.37	1.37
.074	.72	.70	Volume 9 ml.			Volume 8 ml.		
.082	.75	.73	.098	1.05	0.76	.079	1.28	0.95

for these constants that give good agreement between the experimental data and the calculated values. The values of the constants for the different antisera are reasonable. As might be expected the value of A increases as the period of immunization of the rabbit becomes longer. The value of k decreases, *i.e.*, a long period of immunization increases the avidity with which antibody combines with antigen. The value for r remains constant throughout and indicates that the number of reactive groups upon the antigen molecule is 6. It would appear therefore that the reaction between egg albumin and its homologous antibody could be explained upon the assumption of monovalent antibody. However this assumption requires values for the dissociation constant k that would

lead to pronounced changes in the amount of antibody combined should the volume be changed. The data given in TABLE 3 shows that a change in volume does not have the predicted effect. Therefore, in spite of the agreement between experimental and calculated values, the course of the reaction in this system cannot be explained by the assumption that the valence of antibody is one.

It was shown[1c] that, although the antibody obtained after the first course of immunization reacted with egg albumin as if it were homogeneous and had a valence of 2, the antibody obtained from later bleedings did not. Evidence was presented which indicated that the antibody in these sera was not homogeneous, and "that in the later stages of immunization antibody is formed which is reactive with a larger number of chemically distinct groupings on the Ea molecule than was the antibody produced in the earlier stages of immunization." The theoretical treatment of the problem given in this paper permits the testing of this assumption.

An egg albumin molecule was assumed to have two kinds of reactive groups reacting independently with different bi-valent antibody molecules. The number of the first kind of group, which stimulated the rapid formation of antibody in the rabbit, was assumed to be 4. The number of the second kind which stimulated slow antibody response, was assumed to be 2. The relative proportions of the two antibodies was considered to be 1.00 to 0.00, after the first course of immunization, .90 to .10 after the second, and .75 to .25 after the third. FIGURE 4 shows that the experimental results are adequately explained by these assumptions.

Although these assumptions are not the only ones that will lead to the numerical agreement between the experimental and theoretical values, they represent the simplest system that will give agreement. The probability of their correctness is increased by the observation ([1c]: TABLE 5) that, if part of the antibody in an antiserum obtained by long immunization is removed by absorption, the remainder of the antibody reacts as if the antigen had a valence of 4.

In the development of the theory up to this point no assumptions have been made as to the cause of the insolubility of the compound between antigen and antibody. The assumption was simply that the compound was insoluble. The relationship between combined antigen and antibody should hold regardless of the factors responsible for the insolubility of the compound.

Two general theories have been proposed to account for the precipitation of the antigen-antibody complex. One theory postulates that the combination of antigen and antibody leads to the formation of particles
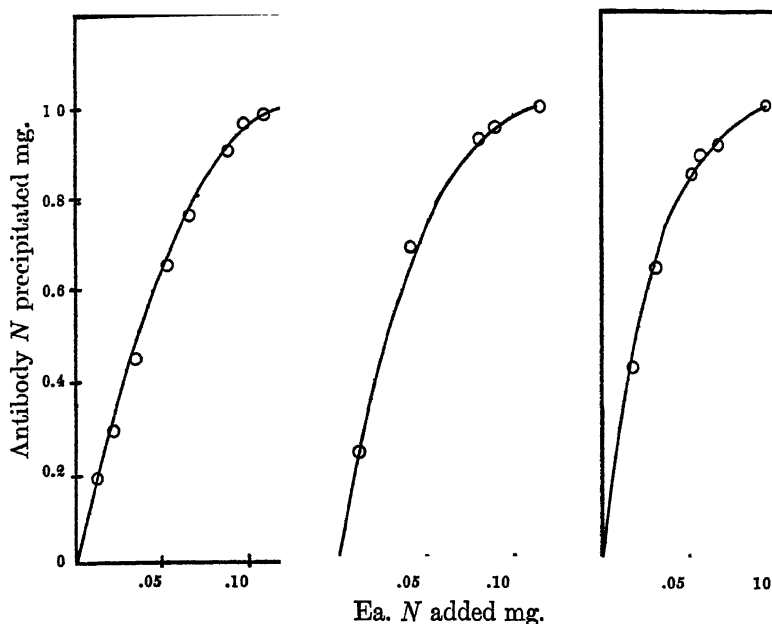
FIGURE 4. Reaction of complex antigen with antibody mixtures. The antigen is assumed to have 4 groups of type A and 2 of type B. The curves are calculated for different mixtures of antibody. 1. 100% A, 2. 90% A, 10% B. 3. 75% A, 25% B. The circles represent experimental values obtained after different periods of immunization of a rabbit with egg albumin.

whose surfaces are more "hydrophobic" than the surfaces of the uncombined antigen and antibody molecules.[4] If sufficient salt is present to reduce the surface charge of these particles below a critical value, nonspecific flocculation and precipitation takes place. The second theory postulates *"specific flocculation"* as well as specific combination.[5] The union of multivalent antigen and antibody should lead to the formation of large aggregates in which alternate molecules of antigen and antibody are linked together to form a lattice-like structure.

It must be recognized that the two theories are interrelated. The same surface forces which prevent non-specific flocculation would also act to prevent the chemical reaction of antigen and antibody groups. The surfaces bearing these groups must come into contact with each other before the reaction can take place. As the "coulomb forces" increase with the size of the aggregates these forces must be reduced below a critical value before specific combination can lead to the formation of large aggregates.

It should be possible to demonstrate mathematically whether or not the "specific aggregation" theory alone is sufficient to explain precipitation.

Let us consider a hypothetical system in which the antigen has N reactive groups all of which are alike and in which the antibody has a valence of 2.   In the presence of an excess of antibody all of the antigen valences may be considered to be in combination.   Part of the antibody molecules in combination will have only one group combined.   It is evident that antigen molecules that are combined only with a singly-bound antibody would form large isolated molecules that would not be part of a large aggregate and so should not be precipitated.   Molecules of antigen combined with a doubly bound antibody molecule may be part of a large aggregate even though most of the antibody molecules upon the antigen are bound with a single valence.

According to the theory presented here the number of antibody molecules which have a single group combined is given by the expression,

$$\frac{(2A - x)x}{2A},$$

where A is the number of antibody molecules present and x is the number of antigen groups in combination and is equal to NB, the number of antigen molecules if k is small.   Since each of these molecules is bound to an antigen group this expression also gives the number of antigen groups bound to a singly bound antibody.   This expression divided by x gives the fraction of the antigen groups so combined, and also the *probability* that any certain antigen group is so combined. $\left(\dfrac{2A - NB}{2A}\right)^N$ equals the probability that all of the groups upon a given antigen molecule of valence N are in combination with a singly bound antibody. $\left(\dfrac{2A - NB}{2A}\right)^N \cdot NB$ is the probable number of antigen groups not combined in a large aggregate and, as each of these groups is combined with an antibody molecule, it is also the number of antibody molecules so combined.   The number of antigen molecules in the aggregates would be

$$\left[1 - \left(\frac{2A - NB}{2A}\right)^N\right]B.$$

As the number of antibody molecules combined has been shown to be $NB - \dfrac{\overline{NB^2}}{4A}$, this number minus $\left(\dfrac{2A - NB}{2A}\right)^N \cdot NB$,

represents the number of antibody molecules in the aggregates.

In FIGURE 5 curves are plotted which show the relative amount of antibody precipitated by various amounts of antigen.   The first curve shows the amount of antibody that would be in combination and the other curves the amount that would be precipitated according to the specific aggregation theory, assuming antigen valences of 6, 4 and 2.   It

Antigen added
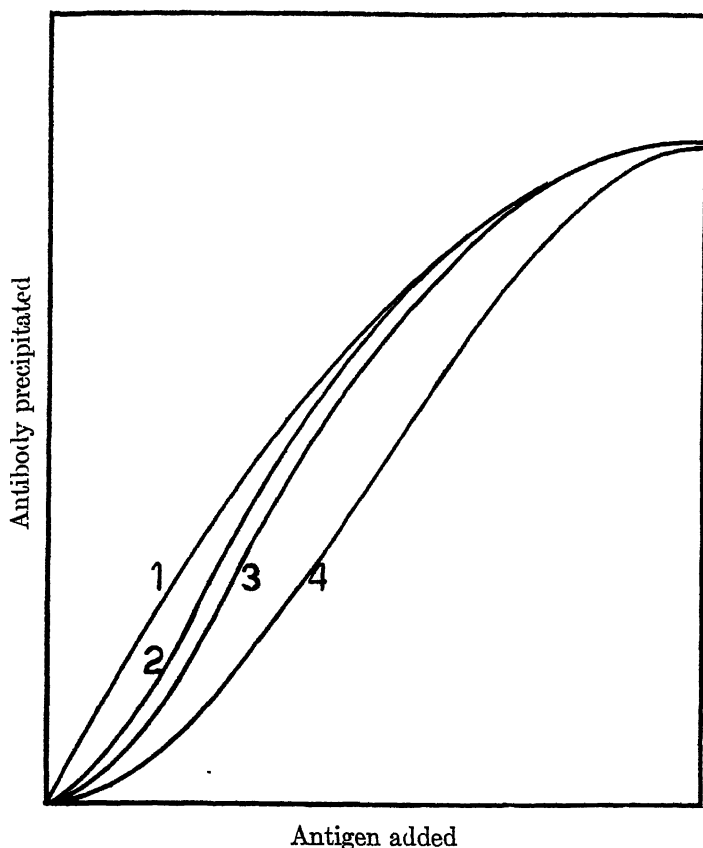
Figure 5. Antibody precipitated according to theory of "specific aggregation." 1. Antibody combined; 2. Antibody precipitated by antigen valence 6; 3. Antibody precipitated by antigen valence 4; 4. Antibody precipitated by antigen valence 2.

has previously been shown that the experimental values obtained for antibody precipitated in anti-protein rabbit systems agree with the values calculated upon the assumption of complete precipitation. The differences between these experimental values and those calculated for the theory of specific flocculation is greater than the uncertainty involved in the determination of the antibody precipitated. It would thus appear that specific flocculation alone is not sufficient to explain the precipitation of the antigen antibody complex. However, the calculated values were obtained upon the assumption that the antibody was homogeneous and that all of the reactive groups upon the antigen were alike. These conditions are probably never encountered in natural systems. It can

be shown that as little as ten per cent of a second antibody reacting independently with the antigen would lead to practically complete precipitation throughout the reaction. Final judgment concerning the adequacy of the theory of specific flocculation cannot be given until simpler systems have been studied in detail.

In contrast to its failure to give satisfactory agreement with the experimental data in the systems in which the antibody was formed in rabbits, the specific agglutination theory, modified in one particular, adequately explains the data obtained in toxin-antitoxin and certain protein-antiprotein systems where the antibody is formed in the horse.

Pappenheimer, Lundgren, and Williams[6] pointed out that "the exceptional character of the antitoxin flocculation reaction lies chiefly in the soluble inhibition zone in the region of antitoxin excess." They suggested that this peculiarity may be due to an "unsymmetrical distribution" of the antitoxic groups upon the surface of the antitoxin molecule. Any assymmetry in the position of these reactive groups which would have any effect upon the reactivity of the groups would be reflected in a difference in the dissociation constants for the reaction of the two groups. Therefore, let it be assumed that an antitoxin molecule has two different reactive groups, A_1 and A_2, which react either with the same or with different reactive groups upon the toxin molecule. If the number of each reactive group on the toxin is equal to or is greater than N, (the maximum number of antitoxin molecules that can be arranged on the surface of the toxin molecule) it makes no difference which assumption is made. In one case the different antitoxic groups would be competing for the same toxin groups and in the other for the same spaces on the toxin molecule. At equilibrium:

$$(A - X)(NT - X - Y) = k_1 VX.$$
$$(A - Y)(NT - X - Y) = k_2 VY.$$

where

A = Number of antitoxin molecules in the system.

T = Number of toxin molecules in the system.

X = Number of A_1 antitoxic groups that have reacted.

Y = Number of A_2 antitoxic groups that have reacted.

N = Valence of toxin. V = Volume.

k_1 = Dissociation constant for A_1 groups.

k_2 = Dissociation constant for A_2 groups.

$(A - X)$ Number of unreacted A_1 groups in the system.

$(A - Y)$ Number of unreacted A_2 groups in the system.

$(NT - X - Y)$ Number of unreacted toxin groups in the system.

From these equations the relationships,

$$Y = \frac{k'Ax}{A + (k' - 1)x} \text{ where } k' = \frac{k_1}{k_2}, \text{ and}$$

$$NT = x + y + \frac{k_1Vx}{A - x}$$

may be obtained.

If X and Y are expressed as fractions of the total antitoxin present, the product XY gives the fraction of the antitoxin with both reactive groups in combination, and XYA the number of antitoxin molecules so combined.

Only those toxin molecules which are combined with at least one anti-toxin molecule that has both groups in combination, can be part of the aggregate and thus be in the precipitate.

The number of molecules of toxin of valence N bound by XYA molecules of antitoxin is given by the formula:

$$T' = \left[1 - \left(1 - \frac{2xyA}{NT} \right)^N \right] T.$$

This formula may be obtained either by an application of the law of mass action to this reaction, or by an application of the law of probability if it is assumed that a perfectly random distribution of the antitoxin molecules exists on the surface of the toxin.

If the proportion of the toxin groups in combination with antitoxin is the same in the precipitate as in the system as a whole the number of antitoxin groups in the precipitate would be

$$\left(\frac{x + y}{NT} \right) T'.$$

The number of antitoxin molecules in the precipitate would be

$$N \left(\frac{x + y}{NT} \right) T' - XYA.$$

These relationships enable one to calculate the number of toxin and antitoxin molecules in the floccules for any value of k_1, k_2 and N. These numbers may be converted into experimental units by assuming that the total antitoxin nitrogen in the system is equivalent to one molecule of antitoxin. The toxin nitrogen equivalent to one valence unit of toxin would then be

$$\frac{\text{Molecular wt. Toxin x \%N} \cdot \text{Valence Antitoxin}}{\text{Molecular wt. Antitoxin x \%N} \cdot \text{Valence Toxin}} \times \text{Total Antitoxin } N.$$

and the nitrogen equivalent to one molecule of toxin, N times this value.

Pappenheimer and Robinson[7] give values for a system containing 0.48 mg. of antitoxin nitrogen. Using their values for the molecular

weight and nitrogen content of toxin and antitoxin the theoretical curve for toxin of valence 4 has been calculated when values of 10^{-8} and 10^{-4} are assumed for the two dissociation constants.

FIGURE 6 compares the theoretical curve with the experimental values and shows that good agreement is obtained up to the point where the precipitate begins to dissolve in an excess of toxin. No attempt has been made to explain the reaction beyond this point.

It would appear that the differences in the reaction of an antigen with antibody formed in a rabbit and the reaction of diphtheria toxin with
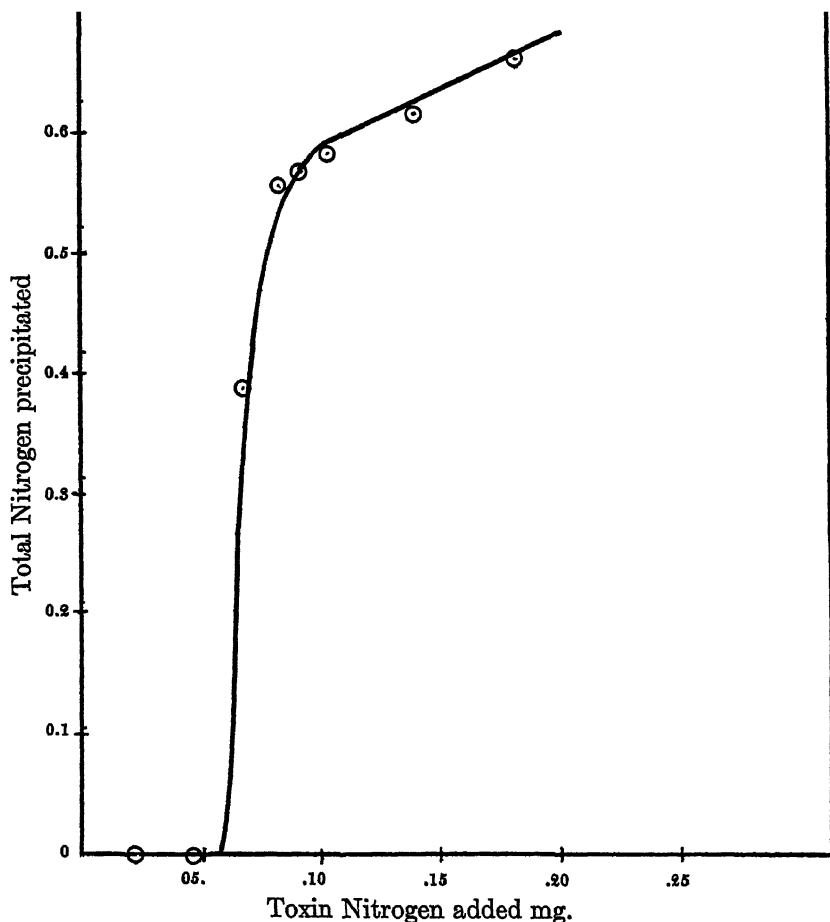
FIGURE 6.   Diphtheria toxin-antitoxin flocculation reaction.   Curve calculated from theory.   Circles represent experimental points.

antitoxin formed in a horse can be adequately explained by assuming that rabbit antibody has two reactive groups which are alike and horse antitoxin has two groups which are different in their reactivity.

SUMMARY AND CONCLUSIONS

It has been shown that the equation,

$$y = 2Rx - \frac{R^2x^2}{A},$$

derived by Heidelberger and Kendall[1a] by an application of the mass law to the precipitin reaction can also be derived by assuming that antibody has a valence of 2 and that there is a random distribution of the antibody groups between the available reactive groups upon the antigen. Although the equation is strictly true only in an irreversible system where all of the groups upon both the antigen and antibody molecules react alike, the effect of dissociation in a reversible system has been shown to be negligible except in the region of the equivalence point if the dissociation constant is small. Great variation can exist in the reactivity of individual antigen groups without producing any great deviations from this formula as long as the antibody is homogeneous. However, if the antibody is not homogeneous significant differences from this formula would be predicted.

REFERENCES

[1] (a) Heidelberger, M., & Kendall, F. E. Jour. Exp. Med. **61**: 563. 1935; (b) Jour. Exp. Med. **62**: 467. 1935; (c) Jour. Exp. Med. **62**: 697. 1935; (d) Jour. Exp. Med. **66**: 229. 1937.

[2] Marrack, J. R. The Chemistry of Antigens and Antibodies. Gt. Brit. Med. Res. Counc., Spec. Rep. Series, No. 230. London. 1938.

[3] Ghosh, B. N. Indian Jour. Med. Research. **23**: 285. 1935.

[4] Eagle, H. Jour. Immunol. **18**: 393. 1930.

[5] Cf. [1a, 2] p. 150, & Pauling, L. Jour. Amer. Chem. Soc. **62**: 2643. 1940.

[6] Pappenheimer, A. M. Jr., Lundgren, H. P., & Williams, J. W. Jour. Exp. Med. **71**: 247. 1940.

[7] Pappenheimer, A. M. Jr., & Robinson, E. S. Jour. Immunol. **32**: 291. 1937.

EQUINE ANTIHEMOCYANIN

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INTRODUCTION

Analysis of antigen-antibody compounds, especially the soluble ones, is facilitated and made more precise and dependable when the antigen (or antibody) contains a structure such as a metallic ion,[1-3] dye,[4] or isotope[5] that is accurately measurable in low concentration. The copper-content, the large molecular size, and the high antigenicity of hemocyanin are properties offering considerable advantages for such analyses.[6] It is desirable to study the characteristics of equine antibody because a large variety of therapeutic sera are derived from horses and bleedings of a size ample for adequate study can be obtained during the course of immunizing individual animals. In 1935 we obtained only a trace of precipitin from a horse immunized with *Limulus* hemocyanin by the Massachusetts Antitoxin and Vaccine Laboratory. Pappenheimer[7] succeeded in producing antiovalbumin in a horse and we have recently obtained a powerful equine antihemocyanin some of whose properties are herein described.

Preparation of the Antigen

Hemocyanin from the blood of *Busycon canaliculatum* was purified by three successive precipitations near the isoelectric point (pH 4.5). The material was dissolved with the minimal amount of alkali, adjusted to about 1 per cent concentration, rendered isotonic by addition of salt, and passed through a Berkefeld filter.

None of the numerous lots of hemocyanin was found to be sterile after Berkefeld filtration, unless a preservative (Merthiolate) had been added. They all contained a coliform bacillus which was cultivable but which in broth would not pass the filter. The hemocyanin thus prepared had the usual nitrogen/copper ratio of about 60, varying somewhat in different lots. From this, and from direct observation of smears, it was apparent that only a very small fraction of the nitrogen in our preparations could have been due to bacteria. Also, before filtration the preparations were centrifuged, which would reduce the number of organisms. This contamination of our antigen must be mentioned although it appears to be unimportant.

Preparation of Antiserum

The Lederle Laboratories very kindly immunized a horse (A633) with the hemocyanin. All injections except the first were given subcutaneously, starting with very small quantities. The amount of the third bleeding was small. Following the fourth bleeding the horse was in such poor condition that he was exsanguinated. Dates and doses are given in TABLE 1.

TABLE 1

HORSE A633

INJECTED WITH HEMOCYANIN OF *Busycon canaliculatum**

Date	Dose	Date	Dose†
1939	*ml.*	*1939*	*ml.*
6/29	1	8/25	20
7/5	1	8/30	Bleeding 1
7/10	2	8/31	20
7/14	3	9/5	20
7/19	4	9/12	Bleeding 2
7/24	5	9/26	40
8/1	5	9/29	15
8/4	5	10/3	15
8/8	10	10/6	15
8/11	10	10/13	Bleeding 3
8/17	20	10/16	Bleeding 4
8/22	20	10/20	Bleeding 5

* The last 3 injections contained 4 per cent protein; all others 1 per cent. Total hcy-N injected was 566 mg.
† Assuming that the horse contained 25 L of "serum" then at the time of the first bleeding 34 grams of circulating antibody had been produced per gram of hemocyanin injected; the corresponding figures for the 2nd and 4th bleedings are 82 and 57 (assumed N-factors: ab, 6.25; hcy, 6.45).

Necropsy revealed a "badly abused liver, about to rupture." Our previous attempt to immunize a horse against *Limulus* hemocyanin also led us to suspect that hemocyanin is toxic to horses. The animal died "with hallucinations." Rabbits, on the other hand, almost invariably tolerate the injections well. Purified *Busycon* hemocyanin does not agglutinate the erythrocytes of horse or rabbit.

The successive bleedings had antibody-nitrogen contents of 0.19, 0.74, 1.15, and 0.94 mg./ml. when redetermined after 15 months with the pooled lot of hemocyanin now being used. Figures obtained when the sera were fresher were but slightly larger; the antibody appears to be pretty stable. A sample of serum from the fourth bleeding, lyophilized immediately, now contains 1.32 mg. *ab-N*/ml. The antibody was

almost entirely in the "pseudoglobulin I" fraction of the serum-proteins, as thrown down with sodium sulphate according to Howe.

The coliform organism grown from the hemocyanin was agglutinated by the immune sera in dilutions up to 1:25 or 1:50 but there was a *decrease* in the agglutinative potency of the sera from the later bleedings, and sera from two other horses not injected with hemocyanin also caused about the same degree of agglutination. Three samples of rabbit-anti-hemocyanin contained no agglutinin for the organism. Thus, this contaminant appeared to have no antigenic significance for the system under investigation.

SEROLOGICAL CHARACTERISTICS

The most prominent feature of the behavior of this antibody is its similarity to diphtheric antitoxin and equine anti-ovalbumin, in that no visible reaction occurred in the region of excessive antibody, in contrast to antihemocyanin or antitoxin from the rabbit. However, the zone in which visible reactions did occur was very much broader than was the case with either of the other equine antiproteins mentioned. This will be apparent from the determinations of nitrogen in precipitates made with various mixtures of antigen and antiserum, shown in TABLE 5 and FIGURES 1, 2 and 3.

Precipitative Reactions

In the zone of equivalence or of slight excess of antigen, no significant difference was observed in the amounts of precipitate obtained after 2 hours at 37°, or at 24 hours or 48 hours in the icebox (TABLE 2).

TABLE 2

SHOWING COMPLETENESS OF PRECIPITATION AFTER TWO HOURS IN ZONE
OF EQUIVALENCE AND OF SLIGHT ANTIGEN-EXCESS.
AVERAGE OF DUPLICATE ANALYSES.

Time	Temp.	Hcy-N added	Serum	Total N	Hcy-N in super.
hr.	*C.*	γ	*ml.*	γ	γ
2	37°	274	1.	610	—
24	6°	274	1.	582	—
48	6°	274	1.	615	—
2	37°	274	0.25	152	9
24	6°	274	0.25	185	6
48	6°	274	0.25	139	10

Several experiments were set up in which the same amounts of reagents in varying volumes (from 1 to 27 ml.) were used. The amount of pre-

cipitate formed from mixtures in proportions corresponding to the mid-point of the equivalence-zone diminished progressively with increasing volume. The decrease seemed to be greater than could be accounted for by simple solubility of the precipitate (TABLE 3).

TABLE 3

INFLUENCE OF CONCENTRATION UPON AMOUNT OF PRECIPITATE
H633₅ 0.5 ml. + 500 γ HEMOCYANIN-N (IN EQUIVALENCE-ZONE)

Volume			Total ppt-N		
ml.					
1.	All		Washed each	1190	Diff.
5.	at		sediment with	977	−213
25.	37°		1 ml. ss after	869	−321
1.	120'	Sed. + 5. ml.	refrigeration	1105	− 85
1.		Sed. + 25. ml.	over night	1041	−149

The solubility in saline of a precipitate made in the region of slight antibody-excess and already washed three times was determined by repeated 3-day extraction in the icebox. It was found that the solubility depended on the total amount of precipitate present, and on the number of previous extractions with saline. The solubility of a pure homogeneous substance should not depend on either of these factors. The solubility was observed to vary from about 40 γ N/ml. saline to 0.1 γ N/ml. The solubility after 6 extractions was decreasing much less rapidly than at first, but had not become stationary. Beyond this point determinations required volumes too large to be practicable (TABLE 4). We may regard the solubility-behavior as evidence of the non-uniformity of the precipitate.

TABLE 4

SOLUBILITY IN SALINE OF THRICE-WASHED PRECIPITATES
(γ N/ml)

Extraction	Volume of extractant	Ml. saline/g precipitate-N
	333 ml.	1660 ml.
1	37.9 γ	21.1 γ
2	16.9	33.6
3	8.2	2.4
4	5.6	2.6
5	3.6	1.1
6	0.4	0.1

Antihemocyanin when heated to 70° for 30 minutes lost its power of precipitating hemocyanin. Such heated serum also greatly retarded the rate of flocculation of hemocyanin with unheated serum, but did increase the amount of nitrogen in the precipitate from such a mixture. In one experiment the increase in amount of precipitate-N was considerably greater than the antibody-N present in the serum before heating. Heated heterologous antisera gave no increase detectable by simple inspection of the centrifuged precipitates. Quantitative data will form the basis of a subsequent report. It is of interest to note the essential similarity of the behavior of this heated serum and the non-precipitating antibody ("univalent"[7, 8]) present in Pappenheimer's horse during the early stage of immunization with ovalbumin. Has the effect of heating been to render the antibody "univalent" or to impair its firmness of union with antigen, perhaps by some reduction in the complexity of its combining group?

TABLE 5

PRECIPITATION OF HEMOCYANIN WITH LYOPHILIZED SERUM A633$_4$
CALCULATED TO 1 ML. OF ANTISERUM. (DUPLICATE ANALYSES).

Hcy-N added	Total N	Hcy-N detected in		Ab-N detected in		"R"[2] ab/ag in ppt.	Mol. R[3] ab/ag
		Ppt.	Super.	Ppt.	Super.		
γ 149	γ 38	γ ?	γ 0	γ	γ +		
196	770	All?	0	574	+	2.93	147
294	1322[1]	All?	0	1028	+	3.50	175
392	1560	All?	0	1168	+	2.98	149
490	1760	All?	0	1270	0	2.60	130
588	1912	All?	0	1324	0	2.26	113
686	1971	All?	0	1285	0	1.87	94
784	2108	All?	0	1324	0	1.69	85
980	2282	All?	Tr.	1302	0	1.33	67
1383	2584	1361	22	1223	0	.89	45
1844	3042	1790	54	1252	0	.70	35
2305	2854	1813	292	941	0	.49	25

[1] One determination lost.
[2] The ratios outside the zone of equivalence are not reliable; see text.
[3] Taking m. w's. as 160,000 and 8,000,000.

Mixtures of serum and antigen containing any considerable excess of either reactant yielded no precipitate. These zonal effects were not observed when the interfacial technic was used. With this technic the stratum of precipitate usually lies above the plane of junction of the reagents, *i.e.*, in the overlying antigen. We have not observed this with rabbit-sera. It could be attributed to the solubility of the precipitate in excess of equine antibody.

On each of the four large bleedings we made a fairly complete study of the relation between composition of precipitate and the proportion in which the reagents were mixed. Some precipitate was obtained even with several times the optimal amount of antigen. The maximal precipitate from a given volume of serum was usually obtained with an amount of antigen somewhat greater than the optimal (Dean and Webb)
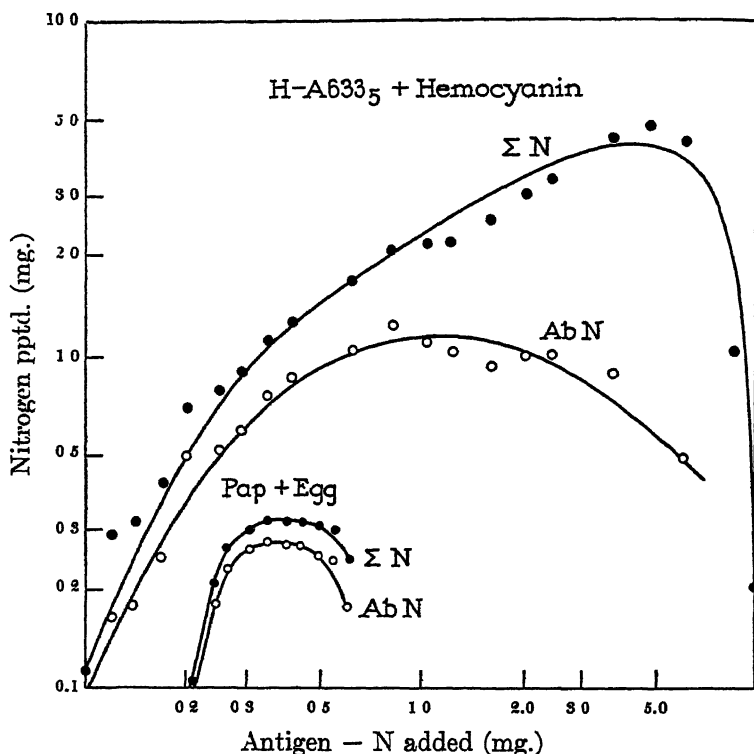
FIGURE 1. Total nitrogen and antibody-nitrogen precipitated from 1 ml. of fifth bleeding of horse 683 (antihemocyanin) and Papenheimer's horse 728 (anti-ovalbumin). (Done while serum was fresher than in FIGURES 2 and 3). Note concavity near top, ignored here, but evident also in FIGURES 2 and 3.

amount, although in the supernate little if any antigen remained (TABLE 5 and FIGURES 1, 2 and 3).

With the possible exception of the equivalence-zone ratios, the proportions of antibody and antigen in precipitates cannot be determined accurately by ordinary serological methods. Soluble compounds containing both antigen and antibody remain in the supernate, so it is impossible to assume that all of one reagent is precipitated and thus to

FIGURE 2. Total nitrogen precipitated from 1 ml of different bleedings of horse 688 by varying amounts of antigen. + or T indicates excess antibody (or antigen) ↓ indicates constant-antibody (right) and constant-antigen optima.
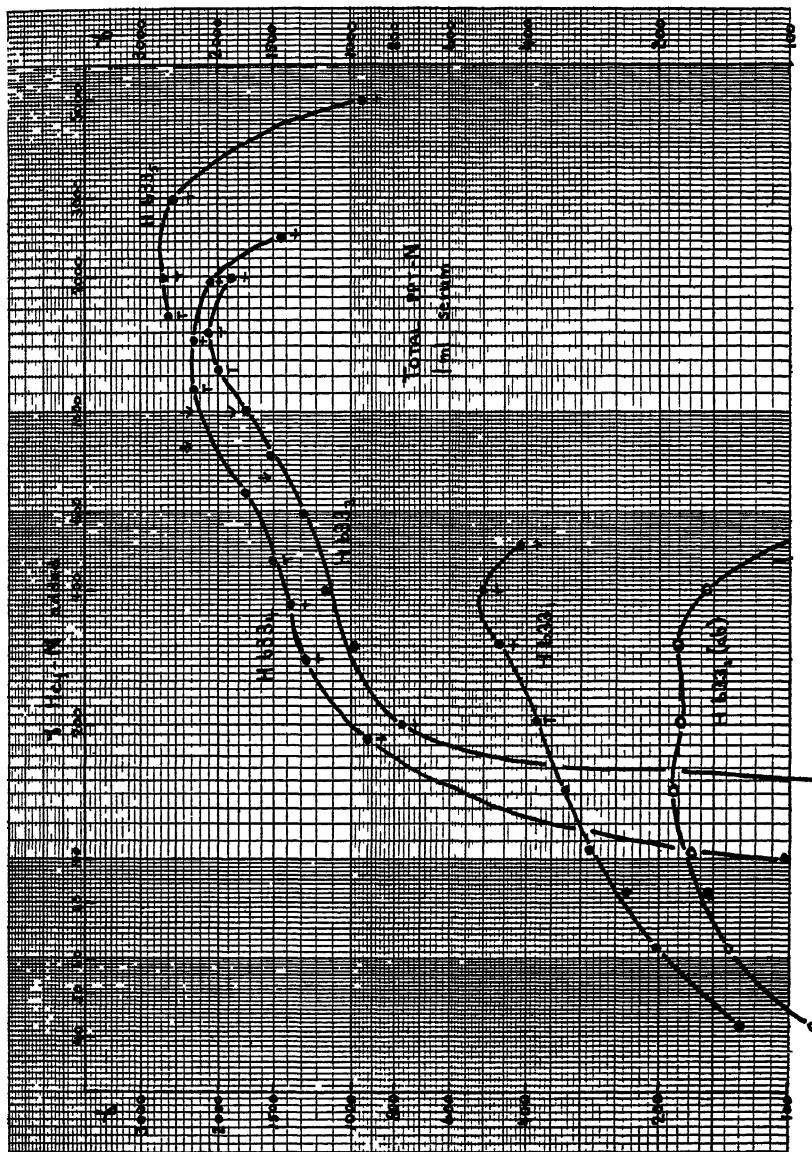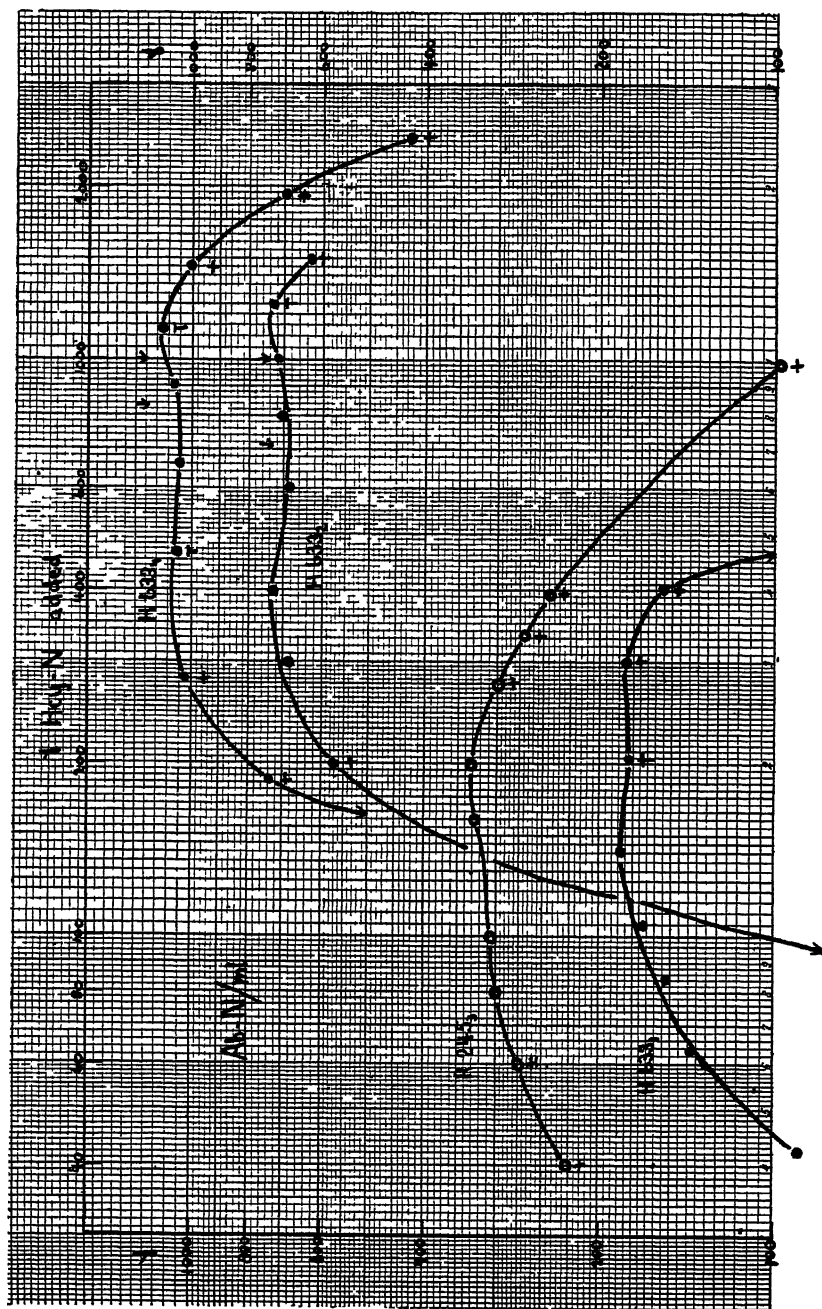
FIGURE 8.   Antibody-nitrogen precipitated from 1 ml. of different bleedings of horse 688 and the third bleeding of rabbit 245, by varying amounts of antigen.

calculate the composition of the precipitate from measurements of nitrogen alone. The "linear-zone" method[9] proposed for determination of residual antigen was not satisfactory for these supernates even when there was a large excess of antigen. Therefore the ratio of antibody to antigen in the precipitates could only be determined by making analyses for copper as well as nitrogen. Preliminary determinations by this method showed that the ratio of antibody to antigen in the precipitate depended on the proportion in which the reagents were mixed, as usual. At the midpoint of the equivalence-zone it averaged about 1:2. From this point the ratio increased or decreased as the proportion of antibody to antigen in the mixtures was made greater or less. At the time of writing, sufficient analyses for copper are not at hand to permit more detailed statement.

The antiserum reacted strongly with hemocyanin from the closely related *B. carica*, and the quantitative data do not suggest any striking difference between the two antigens, although a detailed study of the similarity or possible identity of these two antigens has not yet been undertaken.

Rates of Precipitation

Different lots of hemocyanin varied considerably in the speed of flocculation with immune serum although the amounts of precipitate obtained were not very different. As in other systems, the rate of flocculation depended on the absolute concentration of reagents and on the ratio in which they were mixed. For any concentration of antibody, a certain concentration of antigen gave the most rapid flocculation (Dean-and-Webb optimum), the proportion between these concentrations being the same irrespective of the actual amounts used (TABLE 6). With concentrated reagents, virtually equal speeds of particulation were observed over a moderate range of proportions; with dilute reagents a "dead heat" was less often observed. If the optimum was determined by holding the concentration of antigen constant and varying the antibody (Ramon titration) the optimal-proportions point was not the same. As a consequence of this usual difference between the two optima, it was observed that flocculation in a mixture which was optimal according to the Dean-and-Webb titration could be speeded up by the addition of more antiserum.

Heat of Reaction

An attempt to measure directly the heat of an antibody-antigen reaction was made by Bayne-Jones.[10] It is generally considered[11] that

TABLE 6

"ROUGH" DEAN AND WEBB (ROWS) AND RAMON (COLUMNS) TITRATIONS.
A635₅ AND HEMOCYANIN DILUTIONS 1:1.5 TO THE POWER
ANTIGEN (ϕ = 7.5 mg. N/ml.)

Times of flocculation in minutes

Serum	ϕ	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
ϕ	<.5	<.5	<.5	<.5	<.5	<.5	1	5	34	>90								
1	>90	<.5	<.5	<.5	<.5	<.5	1.5	2	8	73	>90							
2			>90	3	<.5	<.5	1	1.5	4.5	17	>90							
3				>90	<.5	1.5	1.5	1.5	2	6.5	26.5	>90						
4					>90	28	5	5	2.5	5	12	34.5	>90					
5						>90	34	6.5	4	5	6.5	17.5	63	>90				
6							>90	64.5	12	7.5	7	11	24	57.5	>90			
7								>90	47	11	11	12.5	19	40.5	>90			
8									>90	35	17.5	12	16.5	19	48.5	>90		
9											>90	36.5	31	15.5	22.5	63	>90	
10												>90	59	24.5	30	45.5	71	>90

his result is much too high. With Kistiakowsky, *et al.*, we made measurements of the heat evolved when the hemocyanin reacted with serum (TABLE 7). In the region of antibody-excess *where no precipitate is formed,* a value of 3.0 calories per gram of antigen-nitrogen was found. As the molecular weight[12] of the antigen† is 6,800,000, this corresponds to about 3,300,000 calories per mol of antigen. It is believed that this

TABLE 7

HEAT EVOLVED ON MIXING HEMOCYANIN WITH NORMAL AND IMMUNE SERA

	Heat evolved	$-\Delta H$ kcal. x 10^{-3} per gm. hcy-N	$-\Delta H$ kcal. per mol hcy	Calc. $-\Delta H$ kcal. per mol antibody
Series 1.	*Cal.*			
(1) weak hcy + immune serum	+0.350			
(2) weak hcy + normal serum I	0.000			
(1) — (2)	+0.350	2.78	3027	35.6
Series 2.				
(1) weak hcy + immune serum	+0.405			
(2) weak hcy + normal serum II	+0.045			
(3) weak hcy + normal serum III	−0.090			
(1) −[(2) + (3)]/2	+0.428	3.58	3900	45.9
(4) strong hcy + immune serum	+0.144			
(5) strong hcy + normal serum IV	+0.045			
(4) — (5)	+0.099	0.11	116	2.7*

* Calculated for antibody reacting with the second addition of hemocyanin.

value is probably accurate to about 20 per cent. By extrapolation from analyses of specific precipitates, it was calculated that the above result corresponds to about 40,000 calories per mol of antibody. The magnitude of the result would presumably be different when the antibody and antigen were mixed in different proportions, and would probably be different for antigens of different molecular weights on account of the different numbers of specific combining groups.

† Since we began working with hemocyanins the estimates of some of their molecular weights have steadily risen and it is not certain that the upper limit has been reached. It has not always been clearly stated why these revisions have been made. The reader of some of our earlier work may multiply or divide our results by the factor that seems appropriate to him.

TABLE 8

COMPLEMENT-FIXATION

Antigen: Hemocyanin of *Busycon canaliculatum* γ hcy-N

Serum A6335	γ ab-N	2500	1000	500	250	125	63	31	16	8	4	2	1	0.5	.25	.13	.06	.03	.015	.008	.004	Serum control
.04	38	−	4	4	**4***	**4**	**4**	4	3+	2	2	1−	1−	T	0	0	0	0	0	0	0	0 (.16)
.02	19	−	4−	4	4	**4**	**4**	**3**	2+	2	1	T	T	T	0	0	0	0	0	0	0	0 (.08)
.01	9	−	4−	4	4	4−	4−3+	2−	1−	1−	T	0	0	0	0	0	0	0	0	0	0	0 (.04)
.005	4.5	T	4−	4	3+	4−3	1+	T	0	0	0	0	0	0	−	−	−	−	−	−	0	0
.0033	3.1	−	4−	4	1	1−	T	0	0	0	0	0	0	0	−	−	−	−	−	−	0	0
.0025	2.3	T	2	1+	T	T	T	0	0	0	0	0	0	0	−	−	−	−	−	−	0	0
H6331 .04		−	4	4	4	4	4−3−	1	T+	T+	T+	T−	1−	2	3	3+	2	2	2	T+	0	0	T (.08)
R2453 .01	3.3	−	4	4	4	4−	4−3+	3+	3−	3−	3	3+	4	4	4	4	4	4−3	4−3	2−	1−	0	0 (.04)
.007	2.4	−	4	4	4	4−	4−3+	3+	2	2	2	2+	4	4	4	4	4−1	4−2+	4−2+	1+	1−	0	1− (.04)
.005	1.6	−	4	4	4	4−2	3+	2	1−	1−	1−	1−	4−1	4−1	4−3+	4−3+	4−3+	3+	2	1−	T	0	T (.02)
.003	1	−	4	4	4	2	2	1−	T	T	T	T	2+	2	2	2	2	2	1+	T	T	0	0 (.01)
.002	0.7	−	4−	4−1	2	T	1	T	0	0	0	T	1−	1−	1−	1	1	1−T	T	0	0	0	0
.0015	0.5	−	3	2	1	T	0	0	0	0	0	0	0	1−	1−	1	T	T	0	0	0	0	0
Antigen control		T+	T	0	0																		0

Alexin, 2 units. Sheep-cells, 0.1 ml. 10 per cent suspension sensitized with 2 units of lysin. Incubationary period. for fixation = 60′ 37° ; for hemolysis = 30′ 37°. 4 = complete fixation, T = trace, etc. Readings after refrigeration overnight.

* The figures in bold face indicate the mixtures that would give visible precipitation.

Complement-fixation

This system fixed guinea-pig complement strongly. Complete fixation occurred with quantities of antigen and of antibody that entirely inhibited precipitation. It also occurred with very small quantities of antigen; distinct fixation could be obtained with quantities as small as 1γ of antigen-nitrogen; 30γ was the smallest amount giving complete fixation (TABLE 8). This is in marked contrast to the behavior of a weaker rabbit-antihemocyanin ($R245_3$) that gave complete fixation with 0.13γ and definite fixation with 0.008γ antigen-N (1:125,000,000). The rabbit-antiserum exhibited two very distinct zones of fixation, having maxima at amounts of antigen-nitrogen of about 0.25γ and over 1000γ respectively. Serum from the first bleeding of the horse gave similar zones, but the zone of fixation with the small quantities of antigen was not observed in tests on the later bleedings. These zones are considered to indicate the presence of at least two qualitatively different antigenic components or determinants in our preparation of hemocyanin. Lyophilized serum was distinctly more anticomplementary than untreated serum.

Molecular ratios (ab/ag) in mixtures giving complete fixation (TABLE 8) are presented in TABLE 9. These ratios must be considered in attempts to formulate a mechanism of the complement-fixation reactions.

TABLE 9

MOLECULAR RATIO OF ANTIBODY TO ANTIGEN IN MIXTURES GIVING
COMPLETE COMPLEMENT-FIXATION (FROM TABLE 8)

Serum	γ Antigen-nitrogen						
ml.	1000	500	250	125	63	31	16
0.04	1.8	3.6	7.2	14.4	29	57	
0.02	0.9	1.8	3.6	7.2	14		
0.01	0.45	0.9	1.8	3.6			
0.005	0.23	0.5					
0.0033	0.15						

The range of proportions observed to give complete fixation is seen to be 57/0.15 or a 380-fold range. Partial fixation occurred at ratios extending from 1824 to .05, or a 36,480-fold range.
For these estimates the m. w. of antibody was taken as 160,000 and of antigen as 8,000,000.

The heated antiserum already mentioned, which did not precipitate but which did inhibit precipitation, when tested for complement-fixation gave weaker positive reactions and did not inhibit or increase fixation by unheated serum (TABLE 10).

TABLE 10

FIXATION WITH HEATED ANTISERUM

Serum H 633₅	γ Antigen-nitrogen							
70° 30'	1000	500	250	125	63	31	—	0.5
.04 ml.	3+	2	1−	T+	T	0		
.02	2−	1+	T	T	T	0		
.01	T+	T	T	0	0	0		
.005	T	0	0	0	0	0		
Unheated								
.02	4	4	4	4	4	2+	2+	1−
+.02 70°	4	4	4	4	3+	3	2−	1−

Several workers have failed to obtain complement-fixation with horse-antisera.[13] Dean[14] observed fixation by mixtures of crude toxin and antitoxin, but realized that it might "be due to the interaction of other antigens" (than toxin).   Fixation with meningococci[15] and pneumococcal protein[16] have been reported.   We found that Pappenheimer's equine anti-ovalbumin gave definite fixation.   These differences in fixative behavior of equine antisera do not seem readily explainable.

Passive Anaphylaxis

It is also known that most investigators excepting Bailey, *et. al.*[17] have been unable to sensitize the guinea pig passively with equine antiserum.   We also failed to demonstrate passive anaphylaxis in guinea pigs previously injected with any dose of our serum within a wide range (1.0 to 0.005 ml.).   For once the parallelism usually found between the fixative power of a serum and its ability to sensitize passively seems to be lacking.   Typical fatal shock was induced in guinea pigs passively sensitized with rabbit-antihemocyanin containing an amount of antibody within the range of quantities given in the experiments with the equine serum.

SUMMARY

A powerful antiprotein was produced by injecting a horse with hemocyanin.   Its serological behavior resembles strongly that of diphtheric antitoxin and equine anti-ovalbumin.   This supports Pappenheimer's suggestion that equine antiproteins may have definite properties in common, distinguishing them from rabbit-antibodies and from equine anticarbohydrate.   The heat of reaction was measured.   The antiserum, especially when diluted, precipitated hemocyanin over a

relatively narrow range of quantity; it fixed guinea-pig complement strongly, but did not sensitize guinea pigs passively. The serum after moderate heating developed properties resembling those of "imperfect" or "univalent" antibody previously postulated by Heidelberger and by Pappenheimer.

REFERENCES

[1] Wu, H., Sah, P. P. T., & Li, C. P. Proc. Soc. Exp. Biol. **26**: 737. 1929.

[2] Marrack, J., & Smith, F. C. Brit. Jour. Exp. Path. **12**: 182. 1931.

[3] Hooker, S. B., & Boyd, W. C. Jour. Immunol. **23**: 465. 1932.

[4] Heidelberger, M., & Kendall, F. E. Jour. Exp. Med. **62**: 467. 1935.

[5] Schoenheimer, R., Heidelberger, M., Rittengerg, D., & Ratner, S. Meeting of Amer. Soc. Biol. Chem. Chicago. April 18, 1941.

[6] Hooker, S. B., & Boyd, W. C. Jour. Immunol. **30**: 33. 1936.

[7] Pappenheimer, A. M. Jr. Jour. Exp. Med. **71**: 263. 1939.

[8] Heidelberger, M., Treffers, H. P., & Mayer, M. Jour. Exp. Med. **71**: 271. 1940.

[9] Hooker, S. B., & Boyd, W. C. Jour. Gen. Physiol. **19**: 373. 1935.

[10] Bayne-Jones, S. Jour. Immunol. **10**: 663. 1925.

[11] Smith, F. C., & Marrack, J. Brit. Jour. Exp. Path. **11**: 494. 1930.

[12] Eriksson-Quensel, I., & Svedberg, T. Biol Bull. **71**: 498. 1936.

[13] Goodner, K., & Horsfall, F. L., Jr. Jour. Exp. Med. **64**: 201. 1936.

[14] Dean, H. R. Jour. Path. & Bact. **30**: 675. 1927.

[15] Olmstead, M. P. Proc. N. Y. Path. Soc. **15**: 136. 1915.

[16] Lancefield, R. C., & Vollmond, E. Cited in Jour. Exp. Med. **49**: 262. 1929.

[17] Bailey, G. H., Raffel, S., & Dingle, J. H. Amer. Jour. Hyg. **25**: 381. 1937.

STUDIES ON FRESH-WATER BRYOZOA
XII. A COLLECTION FROM VARIOUS SOURCES*

BY

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CONTENTS

INTRODUCTION

The purpose of this article is four-fold. The first objective is to identify and record a small number of Bryozoa collected in Puerto Rico, Guatemala and Utah by several workers. The second objective is to describe a new variety of *Plumatella repens* from Guatemala. The third objective is to add a number of details to the meager morphological data for certain species. These additional data include complete illustrations and measurements of all essential and diagnostic parts. Such measurements are lacking for a number of bryozoan species. The fourth objective is to make some suggestions in regard to the standardization of morphological or taxonomic data for the fresh-water Bryozoa.

The following Bryozoa are described in this paper:—

Class **BRYOZOA**   Ehrenberg 1831
   Order **Gymnolaemata**   Allman 1856
     1. *Paludicella pentagonalis*   Annandale 1916
   Order **Phylactolaemata**   Allman 1856
     2. *Pectinatella magnifica*   Leidy 1851
     3. *Hyalinella vaihiriae*   Hastings 1929
     4. *Plumatella repens*   (Linnaeus 1758).   Lamarck 1816
       a. variety *annulata*   Hozawa & Toriumi 1940
       b. variety *emarginata* (?)   (Allman 1844).   Vangel 1894
       c. variety *typica*   Kafka 1887
       d. variety *osburni* NEW VARIETY

The writers wish to express appreciation to Dr. Henry van der Schalie and Dr. Carl L. Hubbs of the Zoology Museum, University of Michigan, who collected and turned over to us the specimens from Guatemala and Puerto Rico.

COLLECTION DATA

The material came from five collections: the first collection was made in Utah by C. J. D. Brown; the second, third and fourth were made in Guatemala by Henry van der Schalie and Carl L. Hubbs; the fifth was made in Puerto Rico by Henry van der Schalie.

The first collection was made on July 28, 1931, where the Lincoln Highway (U. S. 30) crosses Bear River in Box Elder County, Utah. Here the river banks are steep to the water's edge. The water was alkaline and very murky, carrying a heavy load of silt. Visibility was limited to an inch or so. Almost every stick and stone pulled from the river was covered with a dense growth of *Hyalinella vaihiriae.*

The second collection was made on March 18, 1935, from San Pedro River about 5 miles east of Paso Caballo, Peten Department, Guatemala.

The specimens of the only variety (*Plumatella repens* var. *annulata*) collected in that locality were obtained from depths of 6 inches to 5 feet. The water was warm and the current was rather irregular—swift in the gravel-bottomed riffles and slow in the sand- and mud-bottomed pools.

The third collection was made on March 28, 1935, from a creek at the east end of the south arm of Lake Peten, Peten Department, Guatemala. The creek was almost dried up at that time except for small pockets of water near its banks. The gravel and rubble bottom was overlaid with mud and patches of decomposing terrestrial vegetation. Bryozoa were fairly common on stones and other material in water only a few inches deep. The species found here were *Paludicella pentagonalis* and *Plumatella repens* var. *emarginata* (?).

The fourth collection was made on April 9, 1935, from a small arroyo entering Pasion River from the north, just east of Seibol, Alta Vera Pas, Guatemala. This small stream was about 10 feet wide and 1 to 12 inches deep in the spot where Bryozoa were found. The stream bottom was composed of irregular loose limestone rock, partially covered with sand and gravel. Practically no vegetation grew there but Bryozoa were said to be abundant on the underside of stones. *Pectinatella magnifica* and *Plumatella repens* var. *osburni* were the two forms collected in this locality.

The fifth collection was made on March 13, 1941, from a small stream 3 miles west of Ponce, Puerto Rico. The stream was low and polluted by livestock from nearby fields. The gravel and rubble stream bottom was partly covered by an accumulation of fallen leaves and twigs. Scraps of *Plumatella repens* var. *typica* grew on some of the leaves.

The above ecological data for collections 2 to 5 were secured from the field notes of Dr. van der Schalie.

To our knowledge there are no previously published records of fresh-water Bryozoa from Guatemala, but recently *Plumatella repens* has been recorded by Osburn (1940, p. 467) from Rio Piedras, Puerto Rico.

MORPHOLOGICAL DATA

Paludicella pentagonalis Annandale 1916

Heretofore this species has been reported only once, by Annandale (1916), from Lampam, at the edge of Patalung River near its entrance into the Talé Sap, Singgora Province, peninsular Siam in January 1916. Since then it had not been found in any other locality until collected from Lake Peten, Guatemala on March 28, 1935 by Hubbs and van der Schalie.

Annandale described this species from scraps of material, picturing an

orifice and three zooids and giving three measurements. Our FIGURES 35 to 46 supplement his observations. Annandale mentioned (1916, p. 30) that one zooid was about to produce a young resting bud (hibernaculum), the dimensions of which prove to be smaller than the minimum figures for the Guatemalan specimens. His measurements, as well as additional and more complete ones by the present writers, are included in TABLE 1.

The Guatemalan *Paludicella* fragments were almost entirely material from the basal, attached part of the colony (FIGURES 44 to 46) where the zooecia are more distorted than those from more distal parts of the colony. On the basis of available information and specimens, the outstanding features of this species are: (1) the pentagonal orifice through which the tentacles may be protruded; (2) the distorted shape of the zooecia; (3) the stolon-like sections sometimes found between zooecia; and (4) the general appearance of the hibernacula.

The orifice of a closely related species, *Paludicella articulata*, is unmistakably quadrangular but that of *P. pentagonalis* is not always so sharply pentagonal. The angle at which the orifice is viewed and the degree of tentacular retraction influence the interpretation. When the tentacles are retracted deep into the interior of the animal the orifice looks almost circular (FIGURE 45). When less retracted, the orifice may appear broadly triangular (FIGURE 41); at other times, irregularly quadrangular (FIGURE 44); and when most favorably oriented, definitely pentagonal (FIGURE 42). Actually, the orifice is pentagonal at the free end and becomes circular in outline as it approaches the swollen body of the zooid. In the older zooecia (FIGURE 42), the orifice is reinforced at the angles by narrow chitinous strips which seem stronger at one or two corners than elsewhere.

Tentacles number approximately 16, although the number is difficult to determine accurately in dead specimens (FIGURE 35).

The basal zooecia (FIGURES 44 to 46) are more distorted, dilated and irregularly shaped than comparably located ones of *P. articulata*. The more typical or regular shape is shown in the upper zooid of FIGURE 43.

Relatively inconspicuous short stolon-like tubes connect some of the zooecia (FIGURES 45 and 46). Potts (1884, p. 213) reported, for *Paludicella erecta* (now *Pottsiella erecta*), "meandering cylindrical rhizomes, sometimes of great relative length . . . mostly . . . simple, but . . . sometimes branched." The few stolonate extensions observed in *Paludicella pentagonalis* are all rather short, simple and tubular. Some may be tipped with hibernacula (FIGURE 43) while others merely connect zooids (FIGURE 46).

Hibernacula, which are aggregations of germinative tissue enclosed in a chitinous capsule are found in a small group of fresh-water Bryozoa to which *Paludicella* belongs. Past observers credited these stages with tiding *P. articulata* over winter or similar unfavorable periods. Hibernacula are known to occur between October and April. However, the senior author has found them fairly abundant throughout the summer months also, in collections made in Lake Erie from June to September, 1933. These data are of course for *P. articulata* rather than for *P. pentagonalis*, since the latter species has been recorded only once previously (Annandale 1916). The Guatemalan collection of March 28, 1935 showed a number of well formed hibernacula (FIGURES 36 to 40).

Hibernacula are of several general and intergrading types. Types 1 and 2 occur in *P. articulata*, type 3 in *P. pentagonalis*. Type 1 is external to the zooecia, flattened, often rather pointed at one end, fairly broad in proportion to length, shorter than the parent zooecium and rather irregular in outline. It is pictured or described by Kraepelin (1887, p. 117), Harmer (1913, p. 442 and FIGURE 1), Rogick (1935, FIGURE 11) and others. Type 2 is either external or internal in position, much elongated, spindle-shaped or fusiform, and approaches in size the normal zooecia. This type is represented in collections of Hurrell and Harmer (1913, p. 443 and FIGURES 2 to 10). Type 3 (FIGURES 36 to 39, 43 and 44), from the Guatemalan *P. pentagonalis*, is external in position,

TABLE 1

Measurements of *Paludicella pentagonalis*

	Maximum mm.	Minimum mm.	Average mm.	No. of readings
Zooecial length from septum to septum	1.45	0.80	1.005	23
Zooecial width at widest part....	.40	.155	.289	33
Zooecial width at narrowest point.	.30	.04	.091	33
Zooecial orifice length at side....	.73*	.15**	.296	31
Zooecial orifice outer diameter....	.13	.08	.103	34
Hibernaculum length, longest diameter, including wall........	.34	.20	.256	22
Hibernaculum width including wall	.23	.13	.187	22
Thickness of hibernacular wall ...	.019	.01	.012	10
Annandale's measurements:—				
Zooecial length....	1.2			
Young resting bud length.......			0.1477	
Young resting bud width.......			.102	

*This measurement is of an unsheathed, tentacles-extended orifice but the measurement is just up to the base of the tentacles and does not include the tentacles.
**This was from an incomplete, very young zooecium or else from a broken one, probably the former.

elliptical or more rounded in surface outline than the preceding types and flattened like type 1 in the third dimension. It is smaller than the ordinary zooids. The free surface is curved and quite regular in outline (FIGURES 36 and 38). The attached surface is flat and often has its outline slightly obscured by the cement which attaches the hibernaculum to the substratum (FIGURES 37, 39 and the left, broken one of FIGURE 44). The hibernacula in all cases are opaque, possess a quantity of yolk and germinative material and are enclosed in a firm chitinous capsule which cracks open when germination takes place. Those in the collection seem to be in the developmental rather than in the hatching stage.

The digestive tract is built on the *P. articulata* plan except that funiculi were not accurately discernible in the dead specimens. Annandale encountered the same difficulty with his specimens.

The musculature also is similar to that of *P. articulata* (FIGURE 44).

Lateral buds and branches seem very scarce in *P. pentagonalis* but the colonies examined were all small, mere scraps of material.

Pectinatella magnifica Leidy 1851

This is a common and easily identified species, hence nothing need be said about its descriptive features. Only a single valve from a germinated *Pectinatella* statoblast was found, mixed with *Plumatella* zooecia, from Pasion River at Seibol, Guatemala on April 9, 1935. It is pictured in FIGURE 31. Its measurements are:

Total length of statoblast	0.96 mm.
Total width	.79
Capsule length	.65
Capsule width	.58
Float diameter	.183 to .21
Spine length, average of 6 readings	.168

Hyalinella vaihiriae Hastings 1929

Like *P. pentagonalis*, this species (FIGURES 1 to 9 and 11 to 13) has been found only once previously. It was originally described from a mountain tarn, Lake Vaihiria, in Tahiti by Hastings (1929, pp. 308–310). The present specimens were collected by the junior author in Bear River, Utah on July 28, 1931.

FIGURES 3 to 9 and TABLE 2 present additional diagnostic material for this species and thus supplement the excellent description already given by Hastings. The colony features and the floating statoblasts of the Utah *Hyalinella* (FIGURES 1, 2, and 11 to 13) agree closely with the description of the Tahiti specimens.

Many fresh-water Bryozoa show two types of statoblasts (chitin-encapsuled resting or germinating bodies)—the floating and the sessile types. Some Bryozoa possess one type, others possess both types. *Hyalinella punctata* is reported to have both types (Borg, 1930, p. 120). Hastings (1929) reported finding only the floating type in *H. vaihiriae*, but the Utah specimens of the same species furnished both floating (FIGURES 11 to 13) and sessile (FIGURES 3 to 5) statoblasts.

In addition to these two standard types of statoblasts a few examples of an intermediate type were found in the Utah *Hyalinella* (FIGURES 6 to 8). Very few references to this type occur in the literature. Kraepelin (1892, p. 52 and FIGURE 127) stated that many such intermediate statoblasts were found in a *Plumatella polymorpha fungosa* colony. These statoblasts are considered intermediate in view of the nature of the chitinous lamella (float or annulus) extending peripherally from the capsule (FIGURE 8). In the floating type of statoblast (FIGURES 11 to 13) this annulus is very thick and composed of numerous tiny "air cells" or chambers, while in the sessile type (FIGURES 23 and 24) it is much thinner

TABLE 2

Measurements of *Hyalinella vaihiriae*

	Maximum mm.	Minimum mm.	Average mm.	No. of readings
Free statoblasts				
Total length...................	0.41	0.32	0.356	50
Total width...................	.28	.22	.251	50
Capsule length...	.335	.25	.288	50
Capsule width.................	.25	.20	.223	50
Float				
Dorsal side				
Length.........	.095	.06	.076	21
Width...................	.05	.02	.034	19
Ventral side				
Length...................	.06	.03	.043	35
Width...................	.03	.015	.020	31
Sessile statoblast				
Total length...................	.47	.41	.438	16
Total width....	.40	.32	.351	15
Capsule length.................	.42	.37	.388	16
Capsule width.................	.345	.275	.305	15
Float diameter.................	.055	.02	.030	33
Cement ring diameter, at				
edge of capsule..............	.03	.01	.015	7
Ectocyst tube diameter or width....	.93	.42	.623	31
Ectocyst tube wall thickness........	.07	.05	.054	7
Endocyst tube diameter or width....	.51	.12	.342	26

and far less "cellular" (FIGURE 10). These facts raise the question of whether one type may be transformed into the other under certain circumstances. The intermediate statoblasts found in the Utah specimens, as far as measurements and proportions are concerned, bear a stronger resemblance to the floating statoblasts than to sessile statoblasts.

Identification of *H. vaihiriae* is far less easy than that of the two preceding forms, *Paludicella* and *Pectinatella*, for several reasons. One is its close resemblance to two other species known variously in current literature as *Plumatella repens* var. *fungosa* and *Hyalinella punctata*. The second difficulty is that the diagnostic features of the two similar species are so obscure or confused that identification is not always certain. The Utah specimens are definitely not *P. repens fungosa* because their ectocyst (FIGURES 1 and 2), is distinctly of the *Hyalinella* type rather than of the *Plumatella* type (FIGURE 30). There is a closer relationship between *H. vaihiriae* and *H. punctata* but the difference between them lies in the shape and general appearance of the statoblasts. Kraepelin's (1887, pp. 127–128) description of the two varieties of *Plumatella punctata* (now *Hyalinella*) is inadequate for diagnostic purposes because, after briefly citing distinctive characters for the two varieties, he stated that upon further study, he had found all these features to be variable. Moreover, he implied that the time of year and the amount of nutrition account in part for the differences in size of statoblasts and in number and length of tentacles. That nutrition does have an effect on tentacles has been observed by several workers on a number of occasions; hence, this statement can be accepted. However, well-planned experimentation would be required for ascertaining the effect of season of the year and the exact operation of that factor on statoblast size. This fact is important because the chief distinctions between the various Phylactolaemata are the size and general appearance of the statoblasts.

Plumatella repens var. annulata Hozawa & Toriumi 1940

The Guatemalan representative of this form came from the San Pedro River near Paso Caballo, Peten Department on March 18, 1935. This variety had recently been described by Hozawa and Toriumi (1940, FIGURE 3 and pp. 428–429) from Mu-tan-k in Manchoukuo. Distinctive characteristics are the dimensions of the statoblasts and the "annulation which is formed by the ectocyst thickening in a ring-like manner." This quotation is from a translation which Dr. Makoto Toriumi of the Tôhoku Imperial University graciously furnished with the reprint of his article. In the Guatemalan examples (FIGURE 30), this annulation consisted merely of a stronger reinforcement or stiffening of the angular zooecial

rim and is not particularly conspicuous, hence it was with some hesitation that these were allocated to var. *annulata*.

The following descriptive notes refer to the Guatemalan examples which because they were found growing on an empty mussel valve (FIGURE 26), probably were kept in a dried state from the time of collection. These dried specimens were recently scraped off for study and identification. The nature of the branching or growth habit can be seen in FIGURE 26 and to a lesser extent in FIGURE 30. Septa or dissepiments between the successive zooecia are visible. The tubes branch openly and are adherent along their entire length except at the very tips. Part of the tip is worn away, down to the reinforced rim which naturally would resist wear better than would the other parts. The ectocyst of these apparently young colonies is pellucid yellow and fairly firm. It is not very thick. The polypides have disappeared from the tubes. A striking feature of the dried specimens is the short but prominent keel which broadens out distally into a large V shape as is very accurately shown in the three bigger zooecia in FIGURE 30. TABLE 3 gives a number of measurements for the Guatemalan specimens. The figures given for total and capsule lengths and widths of the free statoblast and for total length and width of the sessile statoblast agree favorably with dimensions given by Hozawa and Toriumi, while the remaining measurements in the table are additional data for this species. Free statoblasts are illustrated in FIGURES 15 and 16 and the sessile one in FIGURE 14.

Plumatella repens var. emarginata (?) (Allman 1844). Vangel 1894

This variety, frequently reported from various parts of the world, is now recorded from Lake Peten, Guatemala, where it was collected on March 28, 1935 (FIGURES 32 to 34). The question mark is here placed after the variety name because it is uncertain whether the Guatemalan colonies are really variety *emarginata*. The reasons for the uncertainty are as follows. First, Allman gave no exact measurements for the statoblasts. Second, confusion exists as to the exact status of two species, *Plumatella emarginata* and *Alcyonella benedeni*, which Allman (1856, pp. 89-91, 104) described in his monograph. Many subsequent workers have considered them synonyms; others have considered them growth forms of the same species. The form reported by Rogick (1940, pp. 189, 200) belongs more definitely to the *A. benedeni* type than do the Guatemalan specimens. Moreover, the statoblasts of the latter are slightly smaller, as comparison of measurements given in TABLE 3 of the present study and TABLE 4 of Rogick's Study IX will show. This poses the problem of whether we are dealing with two distinct varieties or

with only a single vaguely defined one. Thirdly, although the Guatemalan statoblasts agree satisfactorily with the *emarginata* described by Toriumi (1941) the zooecia do not seem to agree too closely. The Guatemalan specimens are further characterized by the following features: branching of the colony open and angular, tubes adherent for the most part, tips not too transparent, tubes colored from a warm yellowish brown to a deep brown, keel prominent in lower half and middle but fades out toward the tip, tubes widely triangular in cross section in middle or lower part, septa present, and numerous sessile statoblasts but few floating ones.

Plumatella repens var. typica Kafka 1887

This variety is represented by a small colony and a statoblast valve collected on March 13, 1941 from a small stream 3 miles west of Ponce, Puerto Rico.

Plumatella repens var. osburni NEW VARIETY

This new variety (FIGURES 17 to 25, 27 to 29 and TABLE 3) was collected in Pasion River near Seibol, Alta Vera Paz, Guatemala, on April 9, 1935. It is named in honor of Dr. Raymond C. Osburn of Ohio State University, whose eminence in the bryozoan field as well as in other fields is well established.

The particular feature on which this variety is differentiated is the nature of its statoblasts, or more specifically, on the measurements of both free and sessile statoblasts, on the shape of the free ones and on the excessive development of the float on the sessile ones. The colonial characters show little that is distinctive. One should refer to FIGURES 17 to 25, 27 to 29 and to the measurements given in TABLE 3 while reading the following description of the new variety.

COLONY. The amount of bryozoan material collected from the rocks was not abundant. At the time of collection the colonies were in the middle stages of development. They form a tan or yellowish tangled mat, fairly open in branching. Evidently enough substratum was available so that much crowding of zooecia was not a necessity. The colonies are scant in appearance and made up of slender zooecia generally adherent except for their tips which are turned upward and are thus partly free.

ZOOECIAL TUBES. The zooecial tubes of var. *osburni* are almost as slender (see TABLE 3) as those of *Fredericella sultana* (Rogick, 1940, TABLE 2, p. 195). Their ectocyst is thin and quite transparent, light yellowish in color, and very little encrusted. Septa between successive

TABLE 3

MEASUREMENTS FOR THREE VARIETIES OF *Plumatella repens*

	Variety *annulata*				Variety *emarginata* (?)				Variety *osburni*			
	Maximum mm.	Minimum mm.	Average mm.	No. of readings	Maximum mm.	Minimum mm.	Average mm.	No. of readings	Maximum mm.	Minimum mm.	Average mm.	No. of readings
Free statoblasts												
Total length	.41	0.285	0.365	51	.44	0.38	0.409	38	0.43	0.40	0.41	5
Total width	.23	.175	.202	51	.24	.19	.213	38	.22	.20	.214	5
Capsule length	.29	.23	.257	51	.30	.25	.274	38	.25	.23	.238	5
Capsule width	.21	.15	.175	51	.21	.17	.192	38	.17	.156	.162	5
Float												
Dorsal side												
Length	.13	.08	.10	35	.13	.10	.114	19	.11	.10	.106	5
Width	.045	.02	.033	35	.05	.015	.035	19	.06	.03	.045	5
Ventral side												
Length	.095	.055	.071	31	.095	.06	.082	16	.105	.09	.097	4
Width	.035	.01	.023	31	.025	.01	.015	16	.035	.026	.030	4
Statoblast thickness	.15	.14	.143	3					.17	.13	.12	1
Sessile statoblasts												
Total length	.41	.335	.373	38	.45	.35	.402	42	.43	.34	.40	35
Total width	.295	.25	.274	38	.34	.22	.278	42	.30	.22	.259	35
Capsule length	.37	.31	.338	38	.43	.32	.369	42	.375	.26	.324	35
Capsule width	.27	.22	.247	38	.315	.19	.253	42	.25	.18	.204	35
Annulus (float) diameter	.03	.01	.018	37	.035	.01	.017	84	.075	.035	.051	35
Statoblast thickness					.15	.10	.126	3	.17	.13	.148	6
Zooecial tube												
Length					3.15	1.8	2.406	27	3.00	2.00	2.557	7
Width, horizontal diameter	.45	.22	.328	50	.43	.25	.342	40	.41	.21	.288	50
Width, vertical diameter of adherent tube					.45	.24	.333	6				
Thickness of tube wall			.01	1	.05	.01	.02	34	.02	.009	.011	24

zooecia are not visible. The basal half to two-thirds of the zooecium is attached to the substratum while the remainder of the tube (the distal tip) is turned upward at a fairly steep angle, away from the surface (FIGURE 27). An inconspicuous keel is present for a short distance along the tube which is generally rounded or elliptical but sometimes even faintly triangular in cross section. Whether the tip or orifice is emarginate or not is difficult to determine because the ectocyst is not encrusted to any great extent. Some zooecia present slight indications of emargination but most do not.

POLYPIDES. Many zooecial tubes contain unusually well-nourished and healthy-looking polypides (FIGURES 27 and 28). These are built along the regular *Plumatella* plan. The digestive tract, particularly the stomach, seems very long. The mouth zone and epistome are tinted a very pale brick red. This is the only bryozoan except *Pectinatella magnifica* in which the writer has observed this condition. As previously stated, it is difficult to count with accuracy the number of tentacles in a dead polypide, so that the number here given, 25 to 33, is approximate.

STATOBLASTS. Two types of statoblasts are found, the floating and the sessile. The former is less numerous than the latter in these colonies. Both are in various stages of development. Some are inside the zooecial tubes, some outside. The free statoblasts are still in the light brown color stage (FIGURES 17, 18 and 25) rather than in the completely matured dark brown condition. A number of the sessile statoblasts are fully mature, some even to the point of germinating or beginning a new colony (FIGURES 28 and 29).

The free statoblasts of var. *osburni* are of approximately the same size as those of other *Plumatella* species of this article. For exact dimensions refer to TABLE 3. They differ slightly from those of other varieties in shape. The free statoblasts of *osburni* have parallel sides and rectangularly curved ends (FIGURES 17 and 18). The float is broad at the poles and the sides on both valves of the statoblast. It encroaches very little on the small, rather elongate capsule. An edge view shows both valves convexly curved (FIGURE 25). Although these free statoblasts closely resemble in general appearance those of *emarginata* pictured by Allman (1856, PLATE 7, FIGURES 7 and 8), the sessile ones do not (present study FIGURES 19 and 24 and Allman's PLATE 7, FIGURE 9).

The sessile statoblasts of *osburni* possess an unusually well developed float, well developed from the standpoint of float width and "cellular" development of the float. The float turns upward at an angle. The cementing substance which characterizes the attached side develops later than the float. Hence, one can easily mistake developing sessile

statoblasts for floating ones, unless one checks the diameters of the capsule which are greater in the sessile statoblasts.   Toriumi (1941, p. 201) shows a similarly well developed float in sessile statoblasts of *P. repens* var. *fruticosa*, but the two varieties are not otherwise comparable.   A sessile statoblast of *osburni*, when germinating, splits much like a free statoblast does, as shown in FIGURES 28 and 29.

DISCUSSION

The difficulties in the identification of fresh-water Bryozoa depend upon a number of factors, some of which have long been known, while others need clarification.   Some of these factors are as follows.

First. there are several classifications for the Plumatellid forms, but no single scheme proves particularly satisfactory or easy to follow.   The chief reasons for this are incompleteness of data on the several species and the variations occurring in the animals themselves.

Second, more definite boundaries need to be established for the species and varieties of *Plumatella* because *Plumatella* is the largest and most important genus of fresh-water Bryozoa.   These boundaries would in time become more clearly defined if the following suggestions were followed: (a) complete illustration of each species, (b) standardization of measurements, and (c) greater knowledge of the life history of the fresh-water Bryozoa, particularly the species of *Plumatella*.

When bryozoan material is small in amount or is in poor condition, complete illustration is out of the question.   But, where material is abundant and in the proper developmental stages, as many of the following features as possible should be adequately pictured:

1. sessile and free statoblasts, from edge and both sides;
2. the colony as a whole, if intact;
3. the nature of the budding;
4. several zooids of various ages, in greater detail;
5. such anatomical features as are of particular significance;
6. larval forms or germinating individuals, if these are not well known;
7. not only the normal but also the abnormal, extreme or variable conditions or features should be pictured;
8. inclusion of a scale of magnification beside the drawings often proves exceedingly useful to workers who might be vitally interested in certain dimensions which the author did not think important enough to record in the text.

In addition to adequate illustration, there is need for complete or standardized measurements of the diagnostic structures in a number of species of *Plumatella*.   In the past, there was little system in recording measurements.   Some workers have given statoblast dimensions in terms of ratios or proportions rather than in metric units.   Others who did use metric units made only limited measurements.   Until more definite boundaries are established for the various species of *Plumatella*

it is the opinion of the writers that every worker should, wherever sufficient material is available, include all possible measurements in a description of any new form or of old forms for which such measurements do not exist.   These standard measurements should include:

1. the various measurable items as given in TABLES 1, 2 and 3 of the present study;
2. the dimensions of the larval forms, if larvae are found;
3. the number of statoblast spines or other similar structures;
4. the number of tentacles, funiculi, etc.

Whenever possible all measurements should be made on living specimens.   Tentacles should be counted and important anatomical details noted while animals are still alive, because preservation generally means retraction of polypides into the zooecial tubes.   This retraction makes observation more difficult and less accurate.   When these standardized data are available for the different species and varieties, perhaps a more satisfactory and acceptable scheme of classification can be worked out.

The third suggestion for reducing the taxonomic difficulties is to increase our knowledge of the life histories of the fresh-water Bryozoa, particularly along these lines:

1. how and what environmental factors affect the color, incrustation, mode of growth, size of colony and size of statoblasts—all important factors in identification;
2. how to culture the Bryozoa so that they may be kept under frequent microscopic observation without being disturbed too much,
3. to rear colonies from hibernacula, larvae and statoblasts of both types throughout the entire life cycle and to observe any differences in colonies resulting from each type of germinative body;
4. observation of Bryozoa under natural outdoor conditions throughout the entire year.

The authors have tried in a small way to meet a few of these problems in the past.   The senior author has attempted to illustrate and measure as completely as possible some of the species, while the junior author (Brown, 1933) has studied the effect of various ecological factors affecting germination of statoblasts.

SUMMARY

1. Seven fresh-water bryozoan species and varieties were collected by Brown, van der Schalie and Hubbs from various localities and sent to the authors for identification.

2. These seven species and varities are:
   - A. Order **Gymnolaemata**
     - 1. *Paludicella pentagonalis*..............from Guatemala
   - B. Order **Phylactolaemata**
     - 2. *Pectinatella magnifica*...............from Guatemala
     - 3. *Hyalinella vaihiriae*..................from Utah
     - 4. *Plumatella repens* var. *annulata*.......from Guatemala

5. *Plumatella repens* var. *emarginata* (?)...from Guatemala
6. *Plumatella repens* var. *typica*.........from Puerto Rico
7. *Plumatella repens* var. *osburni*........from Guatemala

3. One of the seven is a new variety, *P. repens* var. *osburni* and is here described.

4. Three of the seven, *H. raihiriae*, *P. pentagonalis* and *P. repens* var. *annulata*, have been reported only once previously in literature and that was from other localities by their original describers. For these three some new or additional diagnostic data in the form of illustrations and measurements have been added.

5. A third type of hibernaculum was observed in *P. pentagonalis*.

6. A few *H. raihiriae* statoblasts of an intermediate type were found.

7. An outline for the standardization of bryozoological (taxonomic and morphological) data is suggested in the discussion.

8. Several specific vital problems for further research are suggested. These problems confront anyone working on the fresh-water Bryozoa.

REFERENCES

Allman, G. J.
    1844. Synopsis of genera and species of fresh-water Zoophytes inhabiting Ireland. Ann. Mag. Nat. Hist. (1) **13:** 328–331.
    1856. Monograph of fresh-water Polyzoa. London: Ray Soc.

Annandale, N.
    1916. Polyzoa Entoprocta and Ctenostomata. Mem. R. As. Soc. Bengal **6:** 15–35.

Borg, F.
    1930. Moostierchen oder Bryozoen (Ectoprocten). Tierwelt Deutschlands **17:** 25–142.

Brown, C. J. D.
    1933. Limnological study of certain fresh-water Polyzoa. Trans. Amer. Micr. Soc. **52:** 271–316.

Davenport, C. B.
    1891. Observations on budding in *Paludicella* and some other Bryozoa. Bull. Mus. Comp. Zool. Harvard **22:** 1–114.

Harmer, S. F.
    1913. Polyzoa of waterworks. Proc. Zool. Soc. London **1913:** 426–457.

Hastings, A. B.
    1929. Notes on some little known Phylactolaematous Polyzoa and description of new species from Tahiti. Ann. Mag. Nat. Hist. (10) **3:** 300–310.

Hozawa, S., & Toriumi, M.
    1940. Some fresh-water Bryozoa found in Manchoukuo. Rept. Limnobiol. Surv. Kwantung and Manchoukuo **3:** 425–434.

Kafka, J.
    1887. Die Süsswasserbryozoen Böhmens. Arch. f. Naturwissenschaftliche. Landesdurchforschung v. Böhmen. **6:** (2): 1–74.

Kraepelin, K.
  1887. Die deutschen Susswasserbryozoen. Eine Monographie. I. Anatomisch-systematischer Teil. Abhandl. d. natw. Vereins Hamburg 10: 1–168.
  1892. Die deutschen Susswasserbryozoen. Eine Monographie. II. Entwicke-lungsgeschichtlicher Teil. Ibid. 12: 1–68.

Lamarck, J. B.
  1816. Histoire naturelle des animaux sans vertèbres. Paris 6.

Leidy, J.
  1851. Cristatella magnifica n. s. Proc. Acad. Nat. Sci. Phila. 5: 265–266.

Osburn, R. C.
  1940. Bryozoa of Porto Rico. Sci. Surv. Porto Rico and Virgin Islands 16: 321–486.

Potts, E.
  1884. On Paludicella erecta. Proc. Acad. Nat. Sci. Phila. 1884: 213–214.

Rogick, M. D.
  1935. Studies on fresh-water Bryozoa, II. Bryozoa of Lake Erie. Trans. Amer. Micr. Soc. 54: 245–263.
  1940. Studies on fresh-water Bryozoa, IX. Additions to N. Y. Bryozoa. Ibid. 59: 187–204.

Toriumi, M.
  1941. Studies on fresh-water Bryozoa of Japan, I. Sci. Repts. Tôhoku Imp. Univ. (4: Biol.) 16: 193–215.

Vangel, E.
  1894. Daten zur Bryozoen-Fauna Ungarns. Zool. Anzeiger 17: 153–155.

EXPLANATION OF PLATES

All figures, with the exception of FIGURE 26, were drawn with a camera lucida, and hence are not composites but are as nearly like the actual specimens as it was possible to make them. Scales are given for every figure.

The ocular-objective combinations used for the various figures are as follows: 5x-4x, FIGURE 27; 5x-10x, FIGURES 2, 28, 31, 35 and 45; 5x-21x, FIGURE 29; 7.5x-21x, FIGURE 9 and scale Y; 10x-4x, FIGURES 30, 43 and 46; 10x-10x, FIGURES 3, 4, 6, 7, 11–23, 25, 32–34, 36–40, 42, 44 and scale X; 10x-21x, FIGURE 41; 10x-45x, FIGURES 5, 8, 10, 24 and scale Z.

Abbreviations

A, attached surface	M, germinative material	W, rectum
B, barbed processes	N, hibernaculum wall	X, septum
C, colony	O, hibernaculum	Y, small stolon
D, capsule	P, hooks	Z, statoblast valve
E, cement substance	Q, lophophore	IE, statoblasts
F, ectocyst	R, muscles (parieto-vaginal)	AO, stomach
G, edge		IO, substratum
H, endocyst	S, muscles (parietal)	IU, tentacles
I, float	T, muscles (retractor of polypide)	IY, vestigial annulus
J, float "cells"		JE, zooecial rim
K, free surface	U, orifice	JO, zooecial wall
L, funiculus	V, Protozoa	OE, zooecium
		OY, oesophagus

PLATE 1

FIGURE 1.   Part of a preserved *Hyalinella vaihinae* colony. Note the thick, transparent ectocyst, the numerous statoblasts and the mode of branching. This is from a more open section of the colony. In denser sections the zooecial tubes are so crowded that in places their tips closely adhere. Scale *W* belongs with this figure.

FIGURE 2.   An enlarged view of the tips of two degenerate zooecia of *H. vaihiriae*. Note the wrinkling and folding of the thick, transparent ectocyst at the tips. The accompanying scale belongs with this figure.

FIGURE 3.   Side view of a *H. vaihiriae* sessile statoblast which is hanging upside down from a long narrow strip of hardened zooecial wall. The blackest structure in the picture is the cemented base of the statoblast. Note the position and direction of the vestigial annulus. This statoblast and zooecial wall were scraped from a wood fragment. Scale *X* belongs with this figure.

FIGURE 4.   Top view of upper or unattached side of a sessile statoblast of *H. vaihiriae*. Note the markings over the capsule. The vestigial annulus is directed outward and slightly upward. Scale *X* belongs with this figure.

FIGURE 5.   An enlarged view of a part of the upper surface of a sessile statoblast of *H. vaihiriae*. This shows the delicately marked cover which was sometimes observed on the upper unattached surface of sessile statoblasts and which frequently became detached when the statoblast was handled roughly. FIGURE 4 shows a statoblast from which the cover had been accidentally removed. Scale *Z* belongs with FIGURE 5.

FIGURE 6.   The attached side of a very peculiar statoblast of *H. vaihiriae*. This body is peculiar because it looks for the most part like a sessile or attached statoblast but at one end, the lower in this diagram, it has a float which is like that of a free or floating statoblast. This is the intermediate type of statoblast mentioned in the text. The irregular material, gray to black in the diagram, is the cementing substance which attaches the sessile statoblast to the wall. In the specimens themselves this cement ranges from a thin, transparent, colorless layer to a thick, brownish-red substance. A small part of the vestigial annulus is visible at the sides. The float at the bottom is broader on this side than on the reverse side.

FIGURE 7.   The reverse unattached side of the statoblast shown in FIGURE 6. The jagged cement which would be protruding slightly in a few places from beneath the vestigial annulus is not shown here. Note the markings on this side of the capsule. Scale *X* belongs with FIGURES 6 and 7.

FIGURE 8.   A considerably enlarged view of a small sector of the statoblast shown in FIGURE 7. The large light-colored "cells" belong to the true float while the narrow, irregular, light-colored band just below is a part of the vestigial annulus. Scale *Z* belongs with this figure.

FIGURE 9.   A young polypide partially emerged from between the two valves of a germinated *H. vaihiriae* statoblast. The lophophore of the zooid is shown diagrammatically because in the preserved specimen this emerged portion was most indistinct, only the tips of some of the tentacles being seen with any real clarity. This view is of the dorsal statoblast valve. Scale *Y* belongs with this figure.
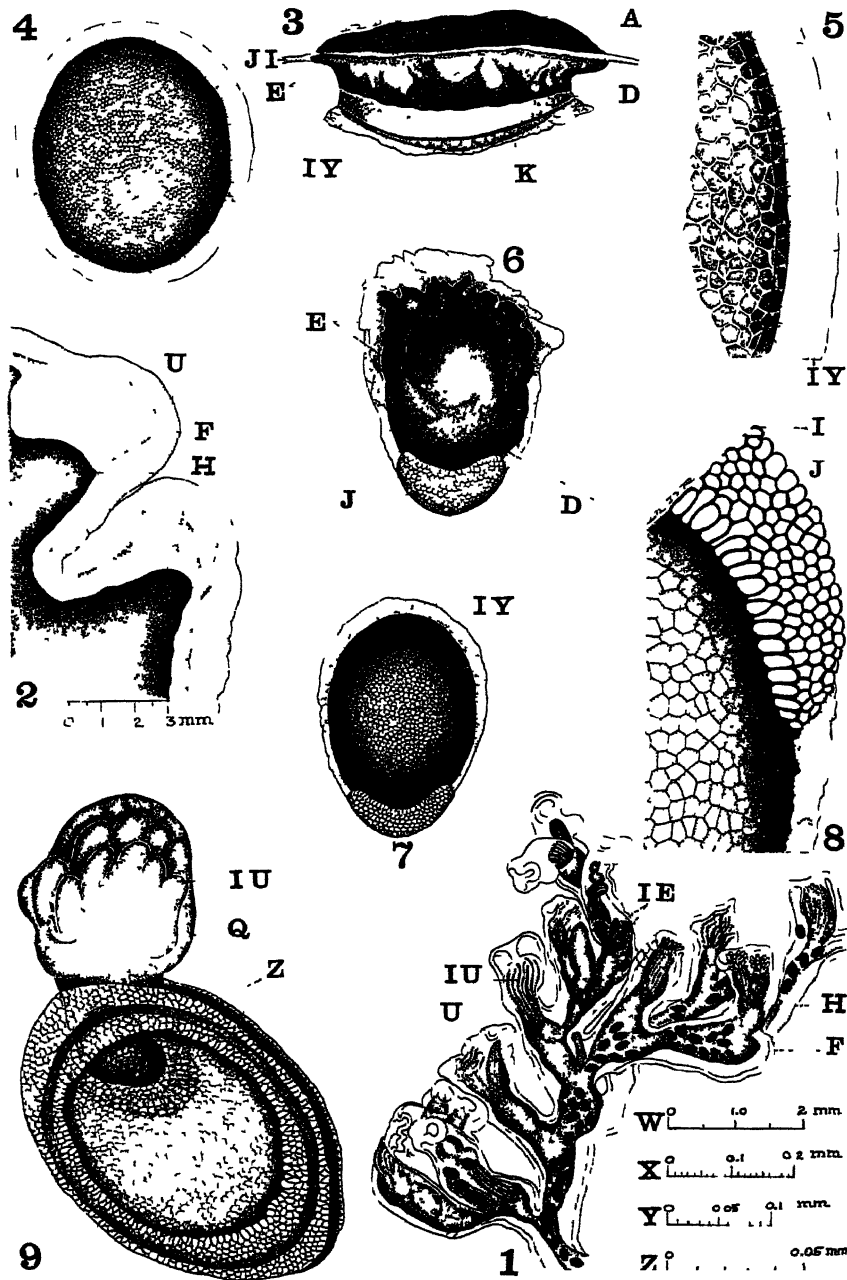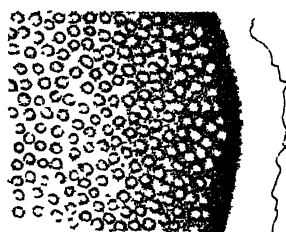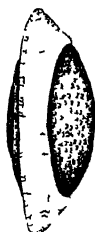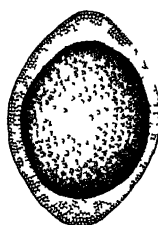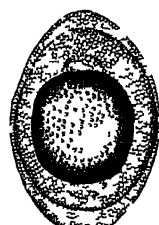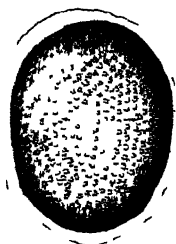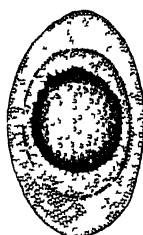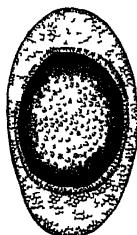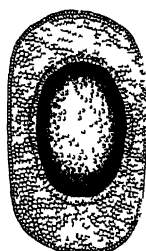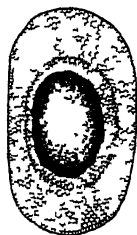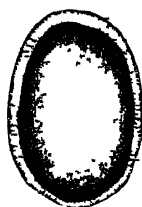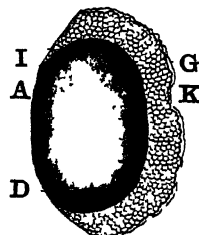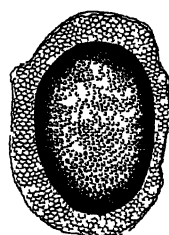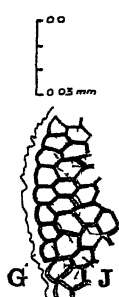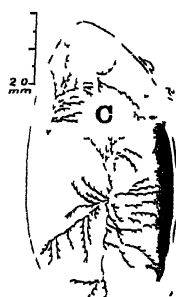
The scale for FIGURE 26 should read 2.0 cm. instead of 2.0 mm.

PLATE 2

FIGURE 10. An enlarged sector of the free surface of *Plumatella repens* var. *annulata* sessile statoblast, showing the mammillated capsule covering and the vestigial annulus. The scale for this figure is placed above FIGURE 24. The whole sessile statoblast is pictured in FIGURE 14.

FIGURE 11. A side view of a floating statoblast of *Hyalinella vaihiriae*, drawn to the same scale as FIGURE 25. The scale appears above FIGURE 25.

FIGURE 12. Ventral side of a *H. vaihiriae* floating statoblast, drawn to the same scale as the preceding figure. This is a slightly more pointed specimen than the average one of this species. Note how extensive is the amount of exposed capsule.

FIGURE 13. Dorsal side of a *H. vaihiriae* floating statoblast, drawn to the same scale as preceding figure. The float encroaches slightly more on the capsule than on the reverse side.

FIGURE 14. View of free surface of a *P. repens* var. *annulata* sessile statoblast, drawn to the same scale as FIGURE 25.

FIGURE 15. Dorsal side of a *P. repens* var. *annulata* floating statoblast, drawn to the same scale as the preceding figure. Note how much the float extends beyond the capsule.

FIGURE 16. Ventral side of a *P. repens* var. *annulata* floating statoblast, drawn to the same scale as the preceding figure.

FIGURE 17. Ventral surface of a *P. repens* var. *osburni* floating statoblast, drawn to the same scale as the preceding figure. Note the very broad ends and the almost parallel sides of the float. Also note the extensive projection of the float beyond the capsule.

FIGURE 18. Dorsal side of a *P. repens* var. *osburni* floating statoblast. This specimen is still somewhat immature, so that the extent of the encroachment of float upon capsule is probably not quite complete. Drawn to the same scale as preceding figure.

FIGURE 19. An immature, developing, sessile statoblast of *P. repens* var. *osburni*. The capsule in this stage is opaque, dark yellowish, and very finely granular. The developing float is very small in extent in this stage. Drawn to the same scale as the preceding figure.

FIGURE 20. A later stage in the development of a sessile statoblast of *P. repens* var. *osburni*, drawn to the same scale as the preceding. The statoblast is slightly tilted so that the float of one side and capsule bottom show. No patches of cementing substance have begun to make their appearance as yet, other than the ring shown around the edge of the capsule.

FIGURE 21. Top or free surface of a *P. repens* var. *osburni* sessile statoblast, drawn to the same scale as the preceding figure and showing the extent of the float. In some specimens the float can be seen to extend slightly over the capsule, as is true of free statoblasts. However, that was difficult to see in this particular statoblast.

FIGURE 22. Side view of a developing sessile statoblast of *P. repens* var. *osburni*, drawn to the same scale as the preceding figure and showing the formation of cement substance on its lower or attached portion. This cement substance is of varying thickness and color. It ranges from a pale transparency to a mahogany color. In this figure the basal part was pale while the reinforcing, constantly enlarging dark patches were reddish brown in color. They seemed to grow from the rim or ring shown in FIGURE 20.

FIGURE 23. Side view of a more mature sessile statoblast of *P. repens* var. *osburni*, showing the excessive development of the basal cement material. Note also the very well developed annulus or float and compare with FIGURE 14. Drawn to the same scale as the preceding figure.

FIGURE 24. An enlargement of the edge of the *P. repens* var. *osburni* sessile statoblast float, showing the irregular border of hyaline chitinous material and the conspicuous "cells." The scale above applies to this figure.

FIGURE 25. Side view of the same statoblast as shown in FIGURE 18. The scale above applies to this figure.

FIGURE 26. Colonies of *P. repens* var. *annulata* growing on the inner surface of a mussel valve. The dotted parts of the colony had been worn away but their location could be traced on the shell. The scale to the left and above applies to this figure.

PLATE 3

FIGURE 27.   Portion of a colony of *P. repens* var. *osburni* showing several zooecia, some of which are partially grown together at the base, although each zooecium is distinct.   A number of Vorticellid Protozoa are seen clustering around some of the orifices.   The scale inserted at right belongs with this figure.

FIGURE 28.   An ancestrula or primary zooid of *P. repens* var. *osburni*, arising from a germinated sessile statoblast.   The two statoblast valves are shown in side view at the base of the zooid.   Drawn to same scale as FIGURE 35.

FIGURE 29.   The reverse side of the basal part of the ancestrula and statoblast valves which were shown in FIGURE 28   Note the extensive development of the float on both valves.   The scale at right, near the label, belongs with this figure.

FIGURE 30.   A portion of *P. repens* var. *annulata* colony, showing the zooecial tubes without any polypide remains, except for the statoblast.   The rim of the upturned zooecial tip is somewhat more strongly reinforced than the rest of the zooecial tube, although the reinforcement is not shown very well in this diagram.   Also note the slight flare or funnel shape just beneath the rim.   It gradually tapers off into a keel   Tube No. 1 shows the shape of this edge rather well.   The scale at left belongs with this figure.

FIGURE 31.   A *Pectinatella magnifica* statoblast valve.   One hook at the edge is broken away, leaving a slight notch in the float.   Drawn to the same scale as FIGURE 35.

FIGURE 32.   Ventral surface of a *Plumatella repens* var. *emarginata* (?) floating statoblast.   Drawn to the same scale as FIGURE 33.

FIGURE 33.   Dorsal surface of statoblast of *P. repens* var. *emarginata* (?).   Scale at right belongs with this figure.

FIGURE 34.   *P. repens* var. *emarginata* (?) sessile statoblast viewed from the attached surface.   Drawn to the same scale as FIGURE 33.

FIGURE 35.   A *Paludicella pentagonalis* zooecium.   It is apparently in a damaged state because its tentacles and part of its internal organs have been forced to the edge of the orifice.   The scale at left belongs with this figure.
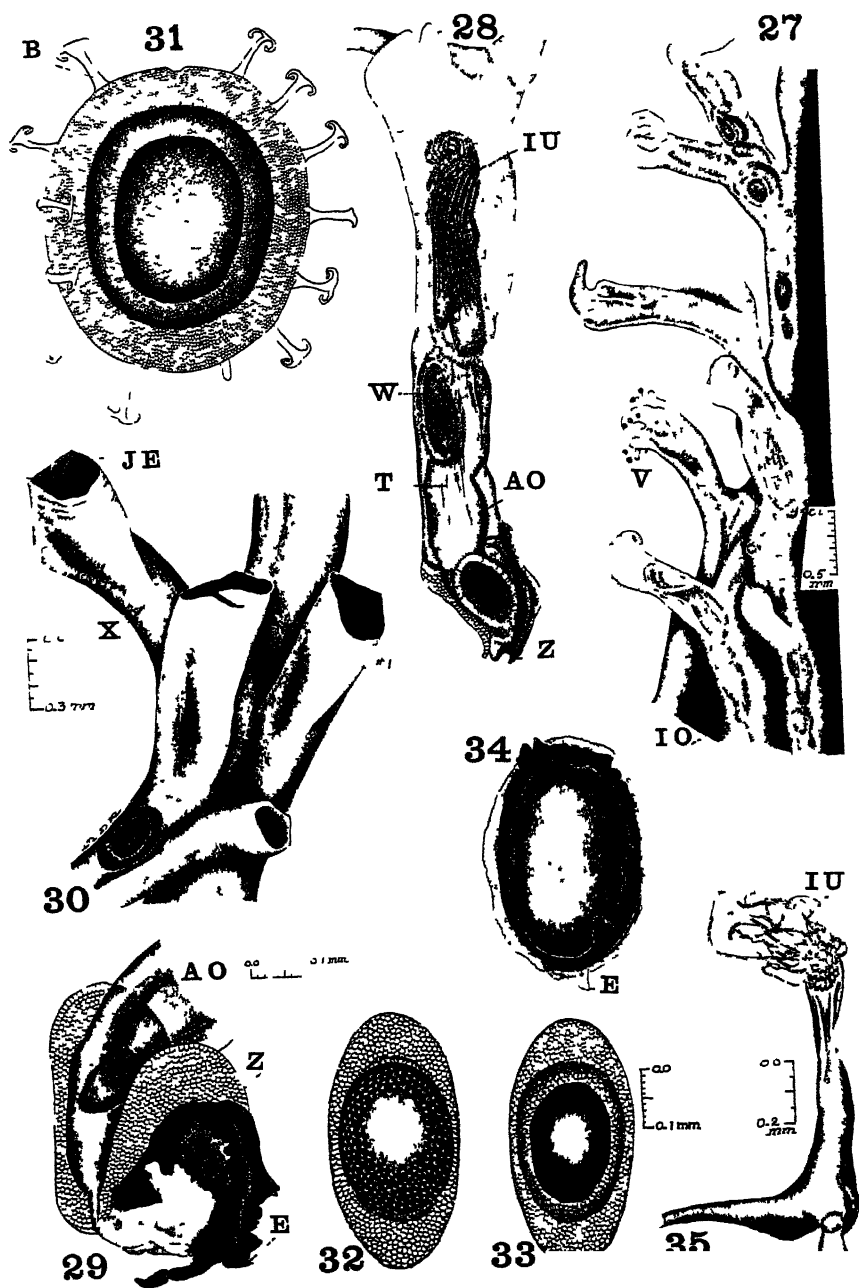
<div align="center">PLATE 4</div>

FIGURES 36 through 40.   Variously shaped hibernacula of *Paludicella pentagonalis*.   Note the heavy chitinous wall of each hibernaculum as compared with the thin walls of the zooecia to which they attach.   All drawn to the same scale (to the right of FIGURE 39).

FIGURE 41.   One view of *P. pentagonalis* orifice.   From this angle it looks triangular although if tilted it would look more pentagonal.   The scale just beneath belongs with this figure.

FIGURE 42.   A better view of the pentagonal orifice of *P. pentagonalis*.   Drawn to the same scale as FIGURE 39.

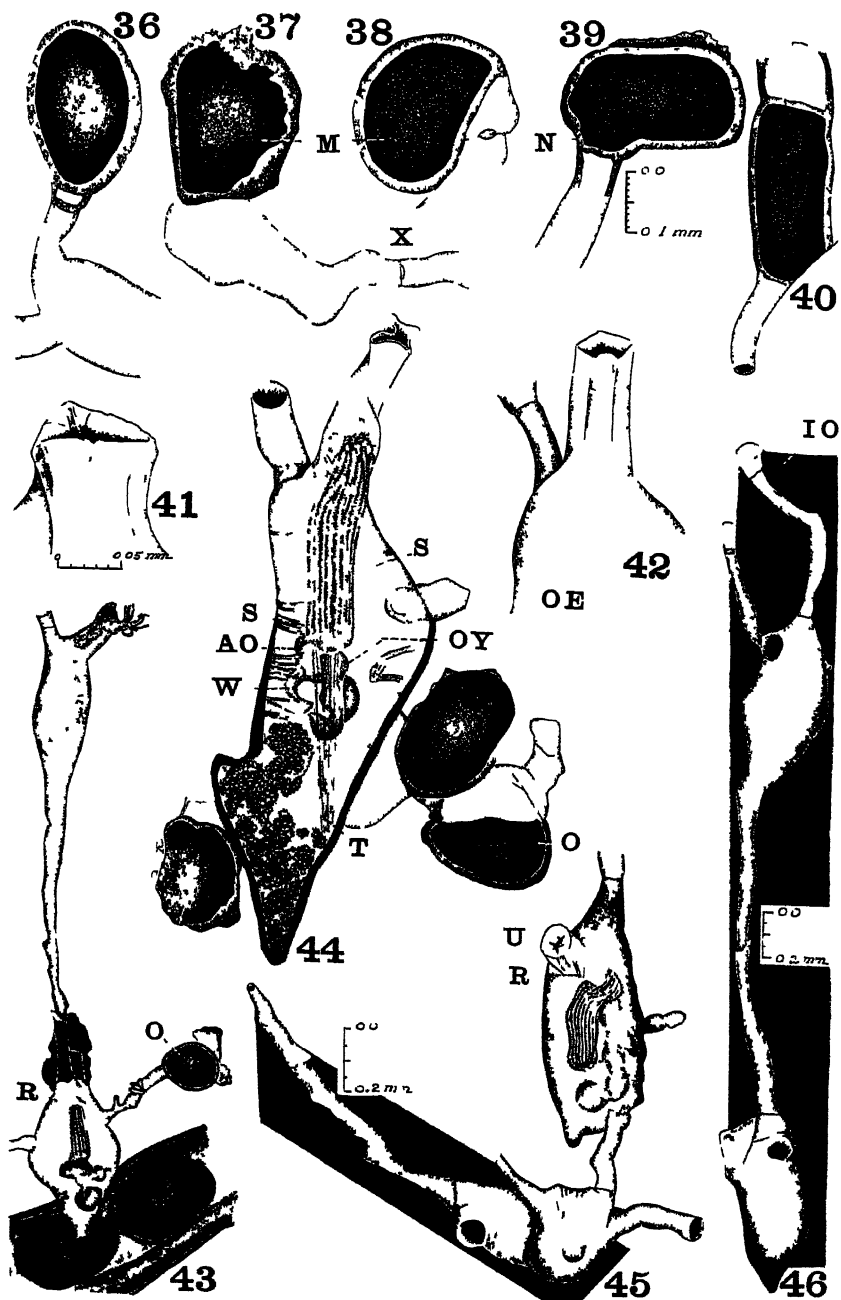FIGURE 43.   Two complete zooecia and a hibernaculum of *P. pentagonalis*.   The basal or ancestral zooecium is shown growing on a portion of *Plumatella repens* var. *emarginata* (?) zooecial tube and statoblasts.   Its basal part had become fastened to one of the two *Plumatella* statoblasts shown in the sketch.   Drawn to the same scale as FIGURE 46.

FIGURE 44.   Several hibernacula and a basal zooecium of *P. pentagonalis*.   The basal part of the zooecium looks somewhat like a hibernaculum.   Note the thickness of the wall in that region and its thinness in the upper part of the zooecium.   It is difficult to say whether this appearance is due to a hibernaculum about to be formed from the basal part, or to the germination of a hibernaculum.   Drawn to the same scale as FIGURE 39.

FIGURE 45.   Irregularly shaped zooecia of *P. pentagonalis*, drawn to the accompanying scale.   The dark strip on which the colony rests represents the substratum. Note the slender stolon-like strip resting on this substratum.

FIGURE 46.   Two empty zooecia of *P. pentagonalis*, drawn to the accompanying scale.   Noteworthy are the long slender extensions of the zooecial base or rather the basal part of the zooecium.   Sometimes septa occur, making these extensions into small stolons.   The dark strip or background represents the substratum, which is diagrammatically shown.

AN ANALYSIS OF THE LOCOMOTION OF THE SEAHORSE, *HIPPOCAMPUS*, BY MEANS OF HIGH SPEED CINEMATOGRAPHY*

BY

C. M. BREDER, JR.† AND H. E. EDGERTON‡

CONTENTS

INTRODUCTION

The forces produced by the vibratory fins serving to propel the sea-horse, *Hippocampus*, have never been analyzed carefully. The high speeds at which these fins are vibrated and the consequent difficulties in reducing them to a readily handled condition are largely responsible for this lack of critical analysis of a peculiar and specialized type of fish loco-motion. Schlesinger (1911) discussed such locomotion in general terms. Breder (1926) attempted a study with an improvised stroboscopic device consisting of a rotating shutter before an incandescent lamp, but found that the apparatus was entirely inadequate for the purpose. Other fishes employing apparently similar fin movements have since been studied by Harris (1934, 1935, 1937 and 1938). However, it would be difficult, if not impossible, to apply his methods to the small and delicate seahorses.

The development of high speed motion picture and still photography supplies a new approach and provides a practical tool permitting the detailed study of phenonoma of this type. As in an earlier study on flying fish by Edgerton and Breder (1941), the photography was done by Edgerton and the analysis by Breder. Several specimens of *Hippocampus hudsonius punctulatus* Guichenot, from the vicinity of Engle-wood, Florida, were used. The photographs were taken at the New York Aquarium shortly before its closure, and the studies were continued in the Department of Animal Behavior of the American Museum of Natural History.

SPEEDS OF FIN MOVEMENTS

Motion pictures of swimming seashores were taken at a rate of 300 frames per second with a General Radio Company stroboscopic-light high-speed motion picture camera. The rate was determined by a centrifugal governor. A timing system, which recorded dots on the edge of the film at 1 60 second intervals, made it possible to measure the exact speed at any given time. The variation in rate after initial ac-celeration is less than a few per cent. Two stroboscopic lamps were used, each excited by a ½ microfarad condenser charged with 2,000 volts. A lens aperture of f:4.5 was used. Photographically suitable sequences were studied in detail, their selection being based on the direc-tion and amount of translation of the fish.

A sequence in which a fish moved forward and upward while holding itself perpendicularly gave a very clear reading of the dorsal waves in comparison to the travel of the fish. Tracings of individual frames at stated intervals were made by means of a projection device. Since each

FRAME
NO.

500
300
200
100
0

FRAME   NUMBER
25   30   35   40   45   50   55

B

A
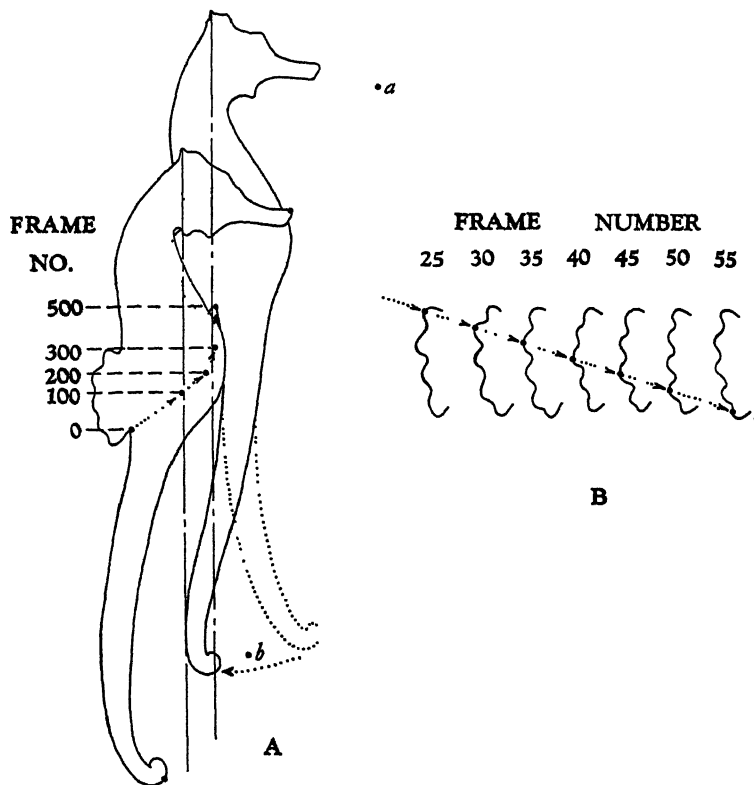
FIGURE 1.   Dorsal fin vibrations with motion forward and upward.
    A. Outlines of *Hippocampus* through 500 consecutive motion picture frames (1⅔ seconds).   Positions of dot on back at posterior base of dorsal indicated at 0, 100, 200, 300 and 500 frame positions. *a*, position snout would have reached if entire fish had followed track of dot on back.   *b*, position tail would have reached if entire fish had followed track of dot on back.   Dotted position of tail shows position tail would have reached if held rigidly, indicating extent of its backward swing.   The two outlines match closely except for this backward swinging of the tail indicating that this accounts in part, at least, for the failure of the snout to reach point *a*.   Note especially the position of the two outlines in reference to the vertical lines drawn from the crest of the head.   The apparent shortening of the snout in the second outline is caused by a slight turning of the head away from the observer.   Note that the angle of the horizontal reactive thrust of the dorsal fin in the first outline is to the left of the vertical and in the second to the right.
    B. Outlines of the margin of the dorsal fin through 30 frames (0.10 seconds).   Positions of dot on one crest and the connecting line indicate travel of wave over fin.   Note that these frame numbers places this detail midway between "0" and "100" frames of "A."

picture interval represents 1, 300th of a second, the calculation of speeds is a simple matter.   FIGURE 1,A shows these tracings through 500 frames or 1⅔ seconds.   The two outlines of the whole fish indicate the extent of its passage upward and forward.   The line passing through a point on the back at the end of the dorsal base indicates the change in direction of travel.   The details of the dorsal fin movement (between frames 0 and 100 where the fish shows its largest horizontal component) is also shown

in a series of 5 frame intervals in FIGURE 1,B. Since the passage of a crest from one end of the dorsal fin to the other covers 30 frames, it follows that one complete cycle of the multiple waves is completed in 0.10 second. In other words, there are ten full cycles per second. Counting those on both sides of the median line, seven crests appear in this sequence. Consequently, an equal number of "planes of pressure" pass over the fin (the internodal parts); that is, 70 such details in the ripple pass down the fin in a second. Each fin ray thus moves from side to side 70 times a second, or completes 35 cycles. As a check against these calculations the sequences were projected at a known rate of speed and the rate of propagation of the wave trains was checked with a stop watch. Projection at 15 frames per second showed that it took almost exactly two seconds for one wave crest to pass over the fin; these measurements thus agreeing with the calculations based on the tracings from separate frames.

Examination of the fin during the more nearly vertical part of the fish's path shows no evident differences in the behavior of the dorsal fin. The pectoral in this otherwise satisfactory sequence could not be discerned with sufficient clarity to establish more than the fact that its waves were passing downward as in the dorsal fin. Sometimes the head of these fishes is moved, changing the angle of attack of the pectoral fins with respect to the body. In the present case the head was held in one position as is seen in the two outlines of FIGURE 1,A. The pendulum-like tail was swung back, changing its position sharply at about the 200th frame, as indicated in FIGURE 1,A.

The actual number of waves employed during this sequence is somewhat greater than those appearing in the still photographs shown in PLATES 1, 2 and 3 or in other sequences, and represents the maximum noted. Toward the end of the sequence, between the 200th and 500th frame, the waves died down, fading out from the posterior end forward and nearly ceasing about the 300th frame; they were then resumed toward the end. This is indicated in FIGURE 1,A by the decreasing distance between the successive points along the line of travel. The late resumption of propulsive effort prevented any effect from becoming evident before the end of the sequence.

In view of the fact that the transparent pectorals were always seen against the dark body of the fish in the motion pictures, they could not be traced accurately from individual frames, in contrast to the pigmented dorsal fin. However, during projection the waves could be seen clearly and in those sequences where both dorsal and pectoral could be seen together it was clear that the waves of each were passing over the fins at

the same rate of speed. This relationship obviously does not always hold, for there is much differential use of the fins in these fishes.

At all times the waves were traveling downward from the top or anterior edge of each fin. None were seen to pass in the reverse direction and it would appear that such motions are used only rarely, chiefly when the fish is attached by means of the prehensile tail.

In both fins it was evident that the wave length was shortened and the amplitude decreased during faster motions and that during the slower movements both the wave length and the amplitude were increased.

No distinctly asymmetrical waves were detected, but what at first appeared to be such were resolvable into variations in amplitude and wave length not occurring uniformly over the entire fin. These occurred most commonly at the beginning or end of active undulations.

It is optically evident that the speed of these waves may vary from zero to the attainable maximum. In actual practice movement of the fins quickly reaches, from a position of rest, a speed that cannot be followed by the eye. How far the speed may exceed the observed rate is uncertain, but it is thought that the limit is less than twice the observed rate. Since the difference in wave length is a matter of angular phase difference and there is no photographic evidence to the contrary, it may be assumed for the present that fewer waves of larger size may pass over the fin more rapidly. Since it has been shown that the individual fin rays may beat back and forth 35 times a second in a phase difference producing seven right and left crests, there seems to be no mechanical reason why they could not all operate in unison an equal number of times a second. This would produce a simple flapping of the entire fin from side to side, a thing that the seahorse has not been seen to do. There is presented in the accompanying plates, however, photographic evidence that the number of complete waves may be considerably reduced.

VECTOR ANALYSES

As a primary step in an effort to understand the origin of the observed resultant motion of a seahorse it is necessary to understand the various possible effects produced by vibratory fins. Given a series of waving rays, the transverse motions of which are responsible for the observed effects, certain limiting features appear:

(1) Each single ray moving from side to side exerts an outward thrust as in FIGURE 2,A. This is the simple and easily understood thrust of a fan. The mechanics of this action have been set forth at length by Breder (1926).

(2) Such rays connected in series by a membrane in which each ray

follows its leader in some uniform phase relationship exerts a force parallel to the axis of the group as indicated in FIGURE 2,B.   The mechanics of this effect have been discussed in various connections by Breder (1926), Breder and Harris (1935) and Harris (1934, 1935, 1937). Similar mechanics involving the whole body of elongate fish have been discussed by Borelli (1680), Pettigrew (1873), Marey (1894, 1895), Du Bois-Reymond (1905), Breder (1926), Gray (1933a, b, c, d, e) and others.

It is thus evident that there are two vectors to be considered in connec-
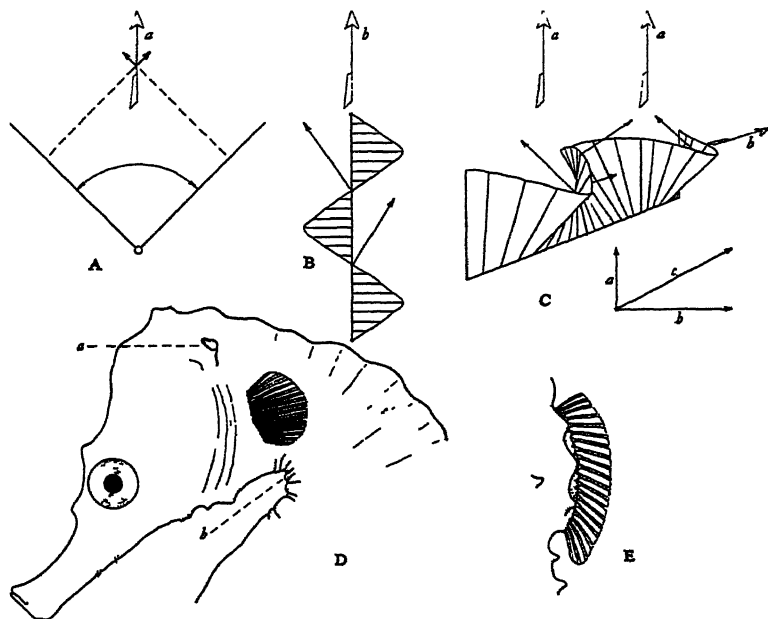
FIGURE 2.   Mechanics of the undulatory fins of *Hippocampus*.

A. Each fin ray describes an arc as shown. Alternately from side to side it exerts pressure normal to its plane as in the dotted arrows, with a resultant in time as indicated by the feathered arrow. A series of these in a row, each at an angular distance from each other, exert a continual pressure in the direction of the resultant.   Connected by a membrane, as in a fin this effect, which is weak because of the small size of the rods, becomes greater and with it appears another effect indicated in FIGURE 2, B.

B. Dorsal view of a series of uniform rods connected by a membrane as in a seahorse fin.   Each ray in this diagram swings 45° to the right and left of a median plane and each follows its predecessor by an angular distance of 11.25°.   Because of the connection of the membrane and the passage of a wave along the series as a unit, pressure is exerted in the direction of the two small arrows, with the resultant indicated by the feathered arrow.

C. Perspective view of such a schematic structure of which "A" can represent an end view and "B" a top view.   Both types of pressure effects are indicated by small arrows and the two resultants by feathered arrows which correspond to those in "A" and "B."   The vector diagram of the two forces *a* and *b* considered as components of the resultant *c* indicate the final effects of this type of locomotor effort if component *b* is larger than *a*.   By modifications of the phase difference, amplitude, *et cetera*, as discussed in the text, either *a* or *b* may be increased at the expense of the other but neither ever disappears entirely.

D. Head of a seahorse showing location of pectoral fin in relation to the gill opening (*a*) and the place in the "neck" region where bending occurs (*b*).   See the plates for various positions of the head, length of the rays, and curvature of the base.

tion with the resultant of the simple wigwagging of fin rays and in the consequent passage of a wave along such a series of identical members. This is indicated in perspective in FIGURE 2,C. Superimposed is the fact that these primary units as found in fishes may vary in two basic ways: (1) physical and (2) behavioristic. These variations modify the resultant and are discussed as to their various implications alone and together in the following pages, for it is in these that reside the answers to many problems in such locomotion.

The physical variables in both the dorsal and pectorals of *Hippocampus* may be summarized as follows:

(1) Distances between rays are not entirely equal although they tend to approximate such a condition.

(2) The length of possible amplitude and the flexibility of rays are not equal; rays are longest and stoutest in the central zone, tapering off gradually anteriorly and more abruptly posteriorly.

(3) The base may not be a straight line, and actually departs from it as shown in FIGURE 2, D and E.

(4) The number of rays may vary somewhat from one fish to another but is always constant in any one specimen. The numbers of dorsal fin and pectoral fin rays reported for the species here studied are as follows:

FIN	MAXIMUM	AVERAGE	MINIMUM	MODE
Dorsal........	21	*19.3*	18	*19*
Pectoral......	19	*16.4*	15	*16*

These figures are based on the data of Ginsburg (1937) and agree closely with the material examined by the writers.

The behavioristic variables may be summarized as follows:

(1) Amplitude of waves may be varied; the vertical vector tends to be increased with waves of large amplitude, and the horizontal vector with waves of small amplitude (FIGURE 2,C).

(2) Variations in phase difference result in wave lengths of greater or less magnitude, and they need not be equal from one part of the fin to another.

(3) Phase difference may vary in each cycle, resulting in asymmetric wave forms (Harris, 1937).

(4) The entire fin may be held at various angles to the body. Thus the dorsal may be deflected far to one side and undulated in that position.

(5) The fin rays are not limited entirely to a transverse movement but have some longitudinal latitude, although such is apparently not used to any noticeable extent in the propulsive efforts. Usually the rays are

not operated in a strictly parallel position but tend to be held somewhat fan-wise. This is especially noticeable at the posterior end of the dorsal fin, as may be seen in some of the plates.

These effects, coupled with the fact that the seahorse normally tends to sink slightly and can change its center of gravity considerably by fairly large movements of the pendulum-like tail element and by movements of the head, give the fish a great flexibility in its otherwise limited locomotor apparatus. The mobility of the neck is especially influential since the pectoral fins are perched anterior to this mobile region and as a consequence change their angle of attack with respect to the rest of the fish when the head is moved (FIGURE 2,D). Aside from these features the seahorse, for all practical purposes, is incased in a rigid armor. The extent of the rigidity and flexibility of the body is clearly indicated in PLATES 1, 2, and 3.

Only males were used for this study. On their ventral surface is located a marsupium for the carrying of eggs; this structure is not involved in locomotion except that, when distended with incubating eggs or young, it becomes somewhat of a handicap to locomotor efforts. The males used were not in this condition, as may be seen most clearly in PLATES 1 and 3. Both sexes possess a tiny anal fin of not more than four short rays. This fin in a male is scarcely ever used, often being buried in the folds of the marsupium. In only one of the specimens studied could the anal fin be seen in the living fish, and in this case its use was not observed. Since this fin is pointed straight down in the females and in the males showing it, it would seem that the only possible function could be to exert a slight lifting force. Of course, the fin might also exert a slight rudder-like action. Ginsburg (1937) has figured no anal fin for the adult of the form here studied, although, as in all known species, the immature males show it. In very small specimens the anal fin is large and prominent. This Ginsburg shows for the young of *Hippocampus hudsonius hudsonius* DeKay; for this form also he figures a large male showing a vestigial anal fin, very like our single specimen noted above. For present purposes the functional significance of this fin may be dismissed with the above remarks. The actual appearance of the dorsal and anal fins, with respect to rays and their relative proportions as shown in this material, may be seen in FIGURE 2, D and E.

From these considerations it is evident that the thrust of such undulating membranous fins is at some intermediate point between the vertical and horizontal components. The condition in the dorsal fin is indicated diagrammatically in FIGURE 3,A. For convenience the vertical component in all figures is indicated by a and the horizontal by b and their

resultant, when shown, by *c*. Since alterations in amplitude and wave length can serve to increase *a* or *b* but neither can be eliminated, as previously indicated, it follows that the maximum effective thrust should appear somewhere between *a* and *b*. Such a condition is indicated in this figure by a dotted line showing the variations in *c* with its angular divergence from *a* or *b*. This has been drawn, for convenience, on the assumption that *a* and *b* are of equal value—an item concerning which there are no exact data.

In the pectorals a similar condition appears, as is indicated in FIGURE 3,B. The vector analysis is identical with that given for the dorsal fin.
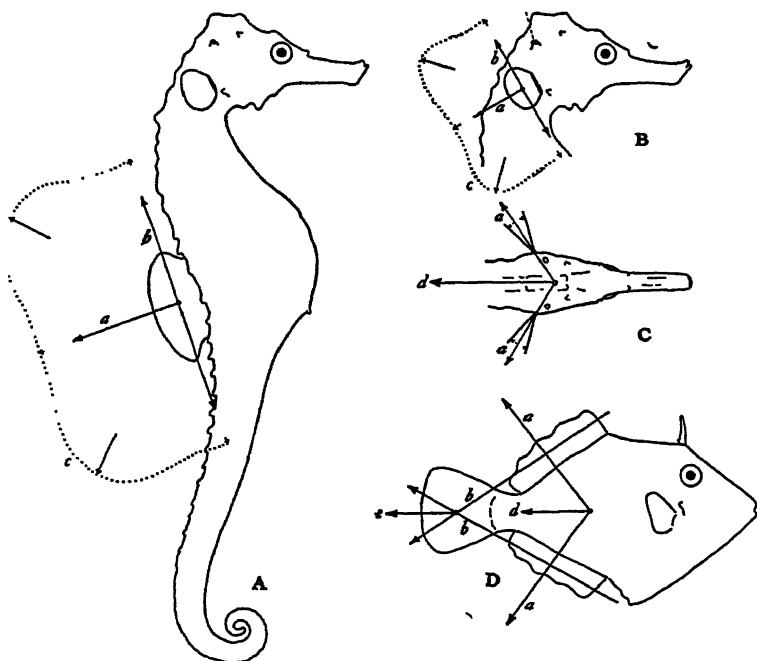
FIGURE 3. Vector analysis of dorsal and pectorals of *Hippocampus.*

A. Dorsal fin with fish in normal slow swimming position. Assuming the components vertical and parallel to the fin to be equal (*a* and *b* respectively) and the waves flowing in either direction, it then follows that the maximum resultants will be along the dotted line *c*. As neither component can be reduced to zero it follows that the increase either of amplitude or wave length necessarily increases each to some extent and the maximum thrust obtainable must be approximately half way between the extremes.

B. Pectoral fin of fish as in FIGURE 3, A. The lettering here is the same and with identical significance.

C. Dorsal view of pectorals. Because the pectorals are paired and not median as is the dorsal it follows that, since they stand out diagonally from the body, their combined components *a* give resultant *d* which, so long as the fins are operated synchronously, falls within a place perpendicular to the back of the head.

D. Vector analysis in *Monacanthus.* Here, the dorsal and anal fins are so arranged that they act as does the pectoral of *Hippocampus* in regard to component *a*, but the components *a* and *b* of both anal and dorsal combine to form two continual backward thrusts, *d* and *e*.

In this diagram the dotted arrow *w* indicates the course of the excurrent water through the gill orifice, a matter to be discussed in another connection. Since there are two pectoral fins a further complication arises here, as indicated by the dorsal view of the head shown in FIGURE 3,C, which is a projection from FIGURE 3,B. Since the fins set at an angle to the head, the two vectors *a* of each fin act in an opposed manner with a backwardly directed resultant *d*, while the two vectors *b* act in parallel as shown for the one in FIGURE 3,B.

It will thus be seen that, if the waves travel from top to bottom in both dorsal and pectorals, the resultant from both will be backward and downward and produce a forward translation of the fish not unlike that shown in FIGURE 1. Since pectoral fins are always paired in any species, an effect similar to that in *Hippocampus* is always obtained. In such forms as the plectognath, *Monacanthus*·(FIGURE 3,D), this is evident without a diagram. However, in this fish which employs similar waves in an undulating dorsal and anal, unlike the seahorse dorsal, the two combine to form components lettered as in FIGURE 3,C. Here too the vectors *b* combine to form a backward resultant *e*. In other words all components unite to form a backward resultant parallel to the long axis of the fish. Harris (1937) expresses this relationship slightly differently with a somewhat similar diagram.

As already mentioned the pectoral fin is involved when the neck of the seahorse is arched and its vectors in various positions with reference to those of the dorsal fin are indicated in FIGURE 4,A. The two extreme positions are about as far as the head normally reaches during locomotion; it is possible for the neck to bend still a little more, although this is generally done only when the fish is resting attached by the prehensile tail. It will be noted that the movement of the pectoral base in neck-bending describes an arc about point *f* which, in view of the interspaces between the plates, cannot be located with any great accuracy. The head position *l* is the same as that of FIGURE 3. The *a* and *b* vectors are indicated only for the dorsal fin and the extreme positions of the pectoral fins. Again for purposes of discussion these vectors are considered equal. The total combined effects are indicated in FIGURE 3,B. The dorsal base is indicated by *D* and the two extreme positions of the pectoral base indicated by *P*, *2* and *3*. The resultant of dorsal and pectoral *a* vectors in positions *2* and *3* is indicated by the numbered and feathered arrows pointing toward the lower left. The *b* vectors of the dorsal and pectorals, parallel to their bases, are not further indicated but their resultants are shown by the two feathered and numbered arrows pointing downward and to the right. For purposes of this diagram it is again
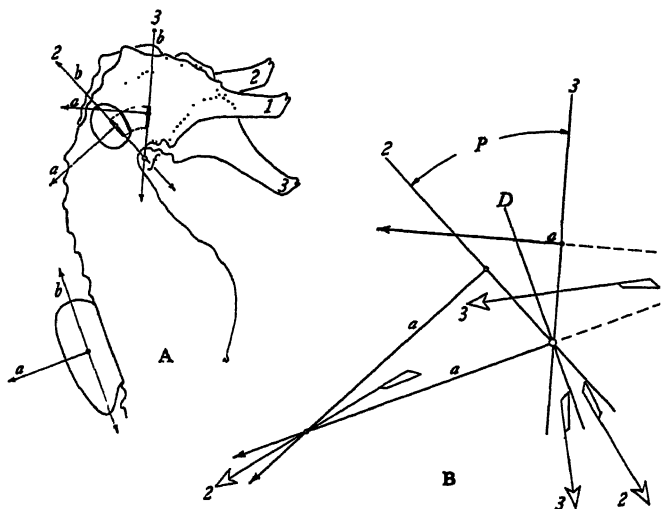
Figure 4.   Effect of head bending on resultants in *Hippocampus*.

A. Position *1* of snout as in FIGURE 3; position *2* with head raised as far as usual; and position *3* with head lowered as far as usual.   The base of the fin rotates about a point, *f*, outside the body of the fish. The position of the dorsal remains relatively fixed.   The components are lettered as in FIGURE 3.

B. Angular relationship of head movement *P*, *2* and *3* represent positions of the pectoral base as in FIGURE 4, A.   *D* represents the position of dorsal base.   Feathered arrows *2* and *3* indicate separately the resultants of components *a* and *b* of the pectoral and dorsal with the former in positions *2* and *3* respectively.   For purposes of this diagram it is assumed that the value of the dorsal and the combined pectoral thrust is equal.

assumed that the *a* and *b* vectors in each case are equal, and further, that the dorsal and the two pectorals, taken as a unit, are of equal value.

A further analysis of the angular relationships of these forces is indicated in FIGURE 5,A.   Lines *V* and *H* indicate the horizontal and vertical in reference to a water surface identical with that assumed in FIGURES 3 and 4 on which this analysis is based.   The *a* and *b* vectors of the preceding two figures are indicated and numbered according to the head positions indicated in FIGURE 4.   The resultants of all *a* and *b* vectors are indicated by the feathered arrows, the numbers referring to the three head positions.   The arrows here fly with the direction of translation of the fish and not with the direction of the force exerted as they do in the previous diagrams; the reasons for this change in the diagram are apparent below.

If these lines of movement are now referred to the original outline on which they were based (FIGURE 3,A) a check may be made to determine the reasonableness of these arbitrary values.   FIGURE 5,B shows this with the feathered arrow indicating the direction of travel.   This is clearly a path that seahorses frequently take, as reference to FIGURE 1

FIGURE 5. Results of vector analysis.

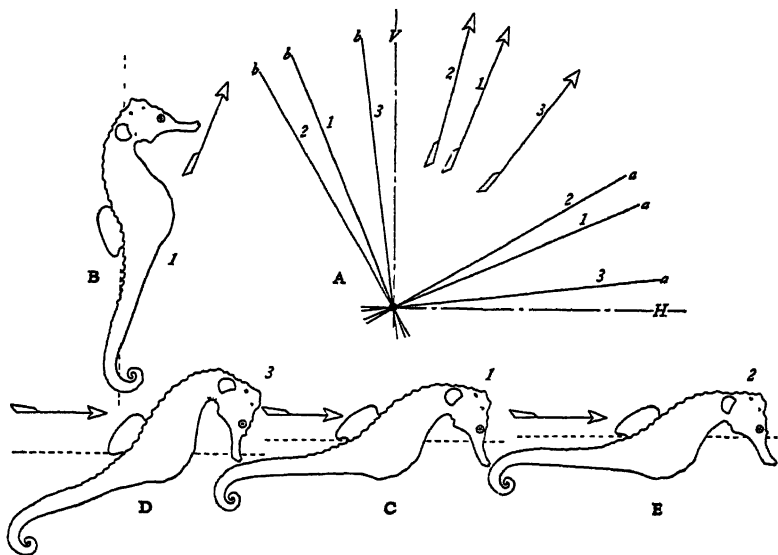A. If the resultants of FIGURE 4, B be again combined to form a single resultant, the lines along which a seahorse should travel by these means alone then could be represented by this diagram of paths of translation. *H* and *V* represent a horizontal and a vertical line. In making this transformation it has been assumed for purposes of the diagram that the fin components *a* and *b* of FIGURE 4, A are equal in effect and that the dorsal and the pair of pectorals are also equal. It follows then that if this diagram approximates reality it should be possible to orient outlines of a seahorse to correspond to a normal position actually realized in forward locomotion. (The seahorse outlines and vectors in FIGURE 3, A and B and in FIGURE 4, A and B all bear the same relationship to the vertical).

B. Seahorse as in FIGURE 3 and 4 with head in position *1*. The path of movement is indicated by the feathered arrow. This a normal position for rising.

C. The same figure rotated as for travel in a horizontal line.

D. Seahorse with the head bent as in position *3*.

E. Seahorse with head extended as in position *2*. Examined from left to right FIGURES 5, D, C, and E are found to represent common positions assumed by seahorses in forward locomotion increasing in speed from left to right. The lettering is as in the preceeding figures. The dotted lines in back of each seahorse figure represents a vertical or horizontal line passing through a point at the posterior base of the dorsal.

will indicate. This outline was originally based on a photograph, the head only of which is used as PLATE 4,B. The fish was actually traveling in about the direction here calculated from theoretical considerations. When seahorses are traveling in a horizontal path at a speed high for them, they incline forward to a considerable extent. If the direction of the arrow of FIGURE 5,B is rotated to the horizontal the fish assumes a position indicated by FIGURE 5,C. This is extremely close to a normal position at a moderate swimming speed. If the resultant *3* with the neck arched is treated in a similar manner the position reached is that shown in FIGURE 5,D. This is typical of relatively slow speed at which the fish appears to be searching below for food. At these times the tail is usually curled tighter, presumably to bring the center of gravity into a

position with reference to the fins so that no rotational element is introduced. The resultant 2 treated as above, with the neck stretched out, appears as in FIGURE 5,E. This is associated with full speed and at such times the tail is frequently stretched out, nearly or completely uncoiled. It thus follows that the arbitrary assumption of equal values for the a and b vectors and for the total thrust of the dorsal and of the two pectorals cannot be far from actuality. If the assumed relationships were far from the real ones, it would probably be impossible, by such means alone, to orient the animal in such normal positions with regard to swimming posture.

It should be obvious that use of the tiny anal fin would produce a thrust nearly parallel to the axis of motion, offsetting that of the dorsal fin to a slight extent after the fashion of *Monacanthus* (FIGURE 3,D). The excurrent water which passes nearly vertically from the top of the head acts in opposition and retards forward motion to that extent. This is not as inconsequential as might be imagined. A resting seahorse attached to a branch with its head near the surface will actually raise a little "mound" of water on each expiration. Further, it has been noted that the reactive effect will cause the head to move down, to an extent dependent on the manner in which the fish is attached and the rigidity of the perch. It has been noted under favorable circumstances that the head may bob down a distance about as great as the length of the pectoral base. Other than the impediment to locomotion that this thrust introduces, there are no moments that cannot be readily countered by appropriate differential activity of the fins, which would be of such small magnitude as to be difficult of detection.

Since seahorses are normally slightly heavier than the water in which they swim, they apparently make use of this fact rather than reversing their fin movements to lower themselves for short distances. A seahorse placed near the surface at some distance from the bottom will normally swim down head foremost, but usually at a low angle, as will one that has become lighter than the water and tends to float. Apparently backing motions of the fins are confined to the making of fine spatial adjustments, mostly when the fish is attached.

DISCUSSION

Although it was realized, from earlier attempts at analysis, that the undulations of seahorse fins progressed very rapidly, the actual measured speed of these wave trains was considerably higher than anticipated.

If the movement of a single ray be considered it makes 35 beats per second. Referred to the beats of an entire insect wing, the movement

involved is, in one sense, comparable. According to Wigglesworth (1939), who drew his data from a large number of sources, insects vary from 330 beats per second in *Musca* to 8 in *Saturnia* and *Colias*. Other insects slower than the seahorse, among the Lepidoptera, are *Pieris* with 12 and *Acidalia* with 32; and among the Odonata, *Libellula* with 20 and *Aeschna* with 28. In Hymenoptera, Diptera and Coeloptera the wing-beats are mostly much higher, although the coleopteran, *Melolontha*, shows 46. Hummingbirds have wing-beats as high as 50 per second, according to Stolpe and Zimmer (1939). Blake (1939) gives an average rate of approximately 55 beats for hovering; rates of 49½ to 75 were reported in straightaway flight, with the statement that the birds were hardly under way and presumably move their wings much faster at other times. It is probably fair to state that the beating of the individual rays of seahorse fins is of only a slightly lower order. It thus appears that the seahorse moves individual rays of its fins through a cycle at speeds approximating those of flying animals of comparable size. But these forms with which the seahorse is being compared do not operate in a medium nearly as dense as water. The muscular effort necessary to obtain speeds such as this under water is naturally much greater. An anatomical comparison of the seahorse muscles responsible for these movements with the corresponding musculature of other forms with rapidly moving locomotor elements should be of considerable interest.

Harris (1937) has shown that the characteristic myotome contractions of a swimming eel-like fish have been preserved in the greatly restricted fin movements analogous to those found in the seahorse. Typically, such waves move from front to back and in their transformed positions start from what is morphologically the anterior edge of the fin, although in both cases reverse waves for backing may be brought into play. The large variation in amplitude of such waves employed by different species of fishes using such locomotor means has been discussed by Harris (1937) and figured by him in considerable detail. The great range of amplitude employed by the seahorse at various times may be seen in the accompanying photographs and outlines. The other evident variable, that of wave length, Harris does not discuss, for in the forms studied by him this feature apparently was not conspicious, if detectable at all.

Harris (1937) points out that if the paired pectorals are held out at right angles to the body while employing symmetrical wave forms the *a* components cancel each other and the fish moves upward by virtue of the *b* components. Although we have no photographs to prove this

assumption, it is possible that the seahorse may at times rise vertically by this means further assisted by undulations of the dorsal fin.

Though we cannot deny some degree of asymmetry of wave form, it is certainly of a very small order of magnitude if present at all and is in no way like that described by Harris (1937) for certain percoids. This type of motion may be noted grossly in that the fins during such movement give the optical impression of rowing.

If the amplitude is increased and the wave length is decreased from one end of the dorsal to the other, a displacement of the resultant of the *a* and *b* vectors is obtained. If this increase in amplitude follows the direction of the wave trains the resultant is displaced backward, whereas if it is in a reverse direction the resultant is displaced forward. On the other hand, the amplitude and wave length may be decreased or increased together. This latter condition is to be seen in PLATE 2. The waves are passing from top to bottom of the dorsal; in the same direction the distances between successive wave crests decrease as does their amplitude. The latter is not true for the fin end but this is likely due to structure rather than differential muscular activity. Measurements expressed in percent of length of fin base are as follows:

<div align="center">ITEM</div>

MEASUREMENT	1	2	3	4
½ Wave length	38	31	20	—
Amplitude	24	20	10	16

The items count from the anterior margin of fin, while "½ Wave length" is measured as the projected distance between alternate crests and "Amplitude" is measured from base of fin to height of crest. Both are expressed as percentage of base of fin.

A geometrical study of this photograph and the diagrams, and of preserved specimens, shows that the complete cycle represented in the dorsal fin of PLATE 2 is composed of twelve fin rays. That is, three rays cover the distance from the median plane to the height of one crest. Where the fin is showing the largest amplitude and greatest wave length, the length of the fin rays indicates a phase difference of slightly more than 17°. This angular measurement becomes less in other parts of the fin.

This form of alteration of a simple symmetrical oscillation with the waves traveling downward—i.e., large wave length and large amplitude to small wave length and small amplitude—shifts the resultant higher on the body of the fish because most of the *a* component comes from the upper part of the fin. Whether this becomes a rotational element or not depends on its relation to the center of gravity as controlled by the head

and tail position and by the use of the other fins. With such elements as these entering, it is easy to see that a great many different combinations of vectors may be obtained, their exact effects depending on the particular way in which the fish holds itself and what it is doing with its other fins.

The body formation of the seahorse, together with its various rugosities, forms a distinct impediment to speed. No conceivable locomotor equipment that the energy in the small body of a seahorse could operate would enable it to proceed at other than a slow speed. The entire design is practically the antithesis of the ordinary stream-lined fish form and the locomotor equipment is relatively frail and inefficient. Although lacking both the form and the propelling mechanism for speed, their equipment with its manifold attributes makes for an easy adjustment of fine spatial relationship. This nearly amounts to saying that, given an irregular body with several feeble but versatile propelling elements variously disposed over the surface, it follows that by their differential use any delicate change of position is possible but high speed is unattainable. Stated another way, there is no evident mechanical advantage in the particular arrangements found in a seahorse that might not just as well be obtained by a large variety of other similarly restricting designs. From this it would appear to follow that the basic pattern of the seahorse design is chiefly one of heritage. In the group as a whole, there is a rather large range of physical variation to be found within the taxonomic family Sygnathidae. Considering only the genus *Hippocampus*, which is somewhat artificially restricted, a reference to Ginsburg (1937) gives evidence of the extensive variation within the genus or even within a single species. When such other forms as the abberent *Phyllopteryx* or *Haliichtys* are considered, it would seem that the exact form which these fishes take has little reference to their highly specialized locomotor equipment.

In those forms which possess in addition a caudal fin and a body much more elongate than most of the aformentioned forms, further complications enter. In genera such as *Sygnathus*, *Acanthognathus*, *Doryrhamphus* or *Coelonotus*, the basic locomotor pattern appears to be identical with the above. It is supplemented by a caudal fin used as a very effective rudder which, with the generally more elongate form, is doubtless of considerable value in making abrupt turns. Furthermore, when excited these fishes make use of their tails in the conventional ichthyized fashion, beating them violently back and forth and taking on an anguilliform locomotion in so far as their relatively rigid body encasement will permit. Breder (1926) wrote: "*Synganthus fuscus* when highly excited may lash

its body into anguilliform curves but makes slight progress, the pectorals and dorsal being the chief locomotor organs." This species may, during slow locomotion, arch its body in a more or less S-shaped curve and progress venter forward, after the fashion of a *Hippocampus*. More often, however, it progresses with its body in a nearly horizontal position. Further indication of the lack of close agreement between locomotor function and the size and form of bodily detail is the existence of several genera that combine various body forms with or without prehensile tails in a bewildering variety, and include elongate forms with prehensile tails, such as *Soleganthus*, *Synganthoides* or *Haliichthys*. Still further removed are solenostomids and centriscids. A detailed study of the locomotor characteristics of these types should be highly illuminating.

From general considerations as well as from the above remarks, it should be apparent that when the limiting restrictions imposed by stream-lining, which is to some degree an absolute necessity for the attainment of any notable speed under water, are once sacrificed, the variations that a fish body may take are capable of a much fuller expression. As has been fully discussed by Breder (1926), there are apparently a limited number of types that a fast moving aquatic form may take whether it be shark, dolphin, penguin, ray or ichthyosaur. Only variations within narrow limits are possible without a sacrifice of speed. The full relinquishing of the demand for speed permits a release of restriction on the necessarily interacting parts, so that in the group considered it is not strange to find all manner of combinations of elements, such as are never found in groups in which even moderate speed is a common trend.

This generalization may have a far-reaching significance in an evolutionary sense. Given a form that fits a certain ecological niche in which one elemental requirement is that the animal be capable of a particular speed—to catch its prey or escape its enemies or both—it follows that any mutant form that does not meet these primary needs must fail to survive or must find a completely different niche in which speed is not a limiting factor. The concatenation of events necessary for success in this latter direction is clearly of less frequent occurrence than are those in which the mutant form does not sacrifice speed. This would seem to account in part for the fact that there are, in groups of fish that are fast swimmers, many species which differ much less in functional body form than they do in color, pattern or other details not of a locomotor significance. On the other hand, the forms under consideration show great variation in shape within a species or even an individual, from time to time, but are as a group basically monotonous in color and pattern, running to browns, greens, tans, and dull reds which are to a marked

degree the predominant colors of the background. On the other hand, lack of speed is associated not with just a single non-stream-lined form, but with a whole group of irregular forms, often extending to the growth of seaweed-like appendages which certainly disrupt the outline of the fish. Although it would be rash to say, at this time, that the comparative invisibility of these forms is *their* limiting factor in evolutionary survival, the above remarks certainly would tend to bolster a view that free swimming fishes are mechanically limited in an evolutionary sense while having relatively great freedom of pattern and color. Inversely then, such forms as the Lophobranchs are more limited in color and pattern, providing an essential invisibility amid their normal backgrounds, but in contrast they have relatively great freedom from the mechanical limitations necessary for speed.

Many other groups could be used to add weight to these remarks by providing similar cases or intermediates. For example, blennies or gobies, although more ichthyized, show much physical differentiation of no locomotor advantage. Such forms as the flounders, although rotated through 90°, still retain remarkable speed and in their way a good streamlining associated with the habit of feeding on smaller but rapidly swimming fishes.

Another element is that of absolute size. Seahorses and their kind are apparently limited to small size. That this has nothing to do with their feeding habits is attested by the fact that fishes of many sizes including some of truly gigantic dimensions are able, simply by straining the water through elaborately contrived gill-rakers, to feed on objects as small as those picked up individually by seahorses. However, this restriction in size could well be associated with the locomotor method and lack of streamlining. For mechanical reasons rapid vibration of locomotor parts is limited to relatively small animals, such as insects and small hummingbirds. The weight-length ratio controls the power necessary to move a given surface, increasing the required mass of muscle tissue to impossible proportions as absolute size reaches a certain limiting value. Because of this condition, large birds such as condors and albatrosses must depend more on slow wing beats and soaring. Their problems of getting into the air and attaining flying speed bear this out.

Another factor may be important in limiting the size of seahorses. Beds of seaweed, limited to a fairly narrow littoral band because of the problem of the penetration of light, are clearly restricted in dimensions by solar radiation and turbidity of water. Like any other plant growth they are further restricted by choking each other out in competing for

light, food and the circulation of water.   For this and similar reasons the seahorse environment is comparatively a small-scale affair.   In other words, a large hiding animal needs a proportionally large hiding place for effective concealment.   It is suggestive in this connection that the largest of the seahorses, *Hippocampus ingens* (Girard), lives on the Pacific coast of Mexico and California—a region abounding in gigantic kelps—whereas the smallest, *Hippocampus zosterae* Jordan and Gilbert, lives, in part at least, among the small floating clumps of *Sargassum* in the Atlantic Ocean.   Furthermore, the latter is a common and abundant form, but the former is reported as rare.

SUMMARY

1. Individual rays in the dorsal and pectoral fins of seahorses may oscillate back and forth as fast as 35 times per second.
2. Undulations in the dorsal fin may have as many as seven advancing "fronts."
3. Propagation of waves in dorsal and pectoral fins is normally at the same speed.
4. Differential effects in locomotion may be attained by altering wave length, amplitude and speed of propagation of waves.
5. Positions of the head and tail affect the center of gravity and play an important part in the locomotor control of the fish.
6. Movements of the head change the relationship between the dorsal and pectoral fins, a feature used to control direction and speed.
7. A long list of physical and behavioristic items provides the seahorse with a nicety of control in its spatial relationships.
8. The complete sacrifice of speed with its restricting limitations has made possible a great diversity of body form in such fishes and permits a disregard of the mechanical interlocking of factors found in faster fishes.
9. For mechanical and ecological reasons fishes with such locomotor equipment are apparently limited to a small size.

BIBLIOGRAPHY

Borelli, G. A.
    1680.   Philosophia de motu animalium ex principio mechanico-statico.   Romae. 2 vols.   168 pp.
Blake, C. H.
    1939.   The flight of hummingbirds.   New Engl. Nat. **3**: 1–5.
Breder, C. M., Jr.
    1926.   The locomotion of fishes.   Zoologica **4**: 159–297.

Breder, C. M., Jr., & Harris, J. E.
    1935. Effect of light on orientation and stability of young Plectognath fish. Pap. Tortugas Lab. **29**: 23–36. Carnegie Inst. Washington, Pub. 452.

Du Bois-Reymond, R.
    1914. Physiologie der Bewegung (In Winterstein, H. Handbuch der Vergleicheden Physiologie. Jena). Schwimmen der Fische: 166–185.

Edgerton, H. E., & Breder, C. M., Jr.
    1941. High speed photographs of flying fishes in flight. Zoologica **26**: 311–314.

Ginsburg, I.
    1937. Review of the seahorses (*Hippocampus*) found on the coasts of the American continents and of Europe. Proc. U. S. Nat. Mus. **83**: 497–594.

Gray, J.
    1933a. Studies in animal locomotion. I. The movement of fish with special reference to the eel. Jour. Exper. Biol. **10**: 88–104.
    1933b. Directional control of fish movement. Proc. Roy. Soc. London. (B) **113**: 115–125.
    1933c. The muscular movements of fishes. Roy. Inst. Great Britain **28**: 1–32.
    1933d. Studies in animal locomotion. II. The relationship between waves of muscular contraction and the propulsive mechanism of the eel. Jour. Exper. Biol. **10**: 386–390.
    1933e. Studies in animal locomotion. III. The propulsive mechanism of the whiting (*Gadus merlangus*). Jour. Exper. Biol. **10**: 391–400.

Harris, J. E.
    1934. The swimming movements of fishes. Ann. Rep. Tortugas Lab., Carnegie Inst. Washington Year Book **33**: 251–253.
    1935. Swimming movements of fishes: anatomy and physiology of the pectoral fin. Ann. Rep. Tortugas Lab., Carnegie Inst. Washington Year Book **34**: 84–85.
    1937. The mechanical significance of the position and movements of the paired fins in the teleostei. Pap. Tortugas Lab. **31**: 171–189.
    1938. The role of the fins in the equilibrium of the swimming fish. II. The role of the pelvic fins. Jour. Exper. Biol. **15**: 32–47.

Marey, E. J.
    1895. Movement. Transl. by Eric Pritchard. Internat. Sci. Ser., New York. 323 pp.
    1894. Le Mouvement. Paris.

Pettigrew, J. B.
    1874. Animal Locomotion; or walking, swimming, and flying, with a dissertation on aeronautics. Internat. Sci. Ser., New York. 264 pp.

Ryder, J. A.
    1882. A contribution to the development and morphology of the lophobranchiates, (*Hippocampus antiquorium*). Bull. U. S. Fish. Comm. **1**: 191–199.

Schlesinger, G.
    1911. Über undulatorische Bewegung bei Fischen. Verh. Zool.-Bot. Ges. Wien **61**: 301–322.

Stolpe, M., & Zimmer, K.
    1939. Der Vogelflug. Leipzig, Akademische Verlag. 159 pp.

Wigglesworth, V. B.
    1939. The principles of insect physiology. Dutton, New York. 434 pp.

EXPLANATION OF PLATES

From still photographs at 1/10,000 second by H. E. Edgerton.

PLATE 1.

Hippocampus dragging its tail along the bottom, with large waves in the dorsal fin, few in number, and with small waves in the pectoral, many for that fin.

BREDER AND EDGERTON *HIPPOCAMPUS*
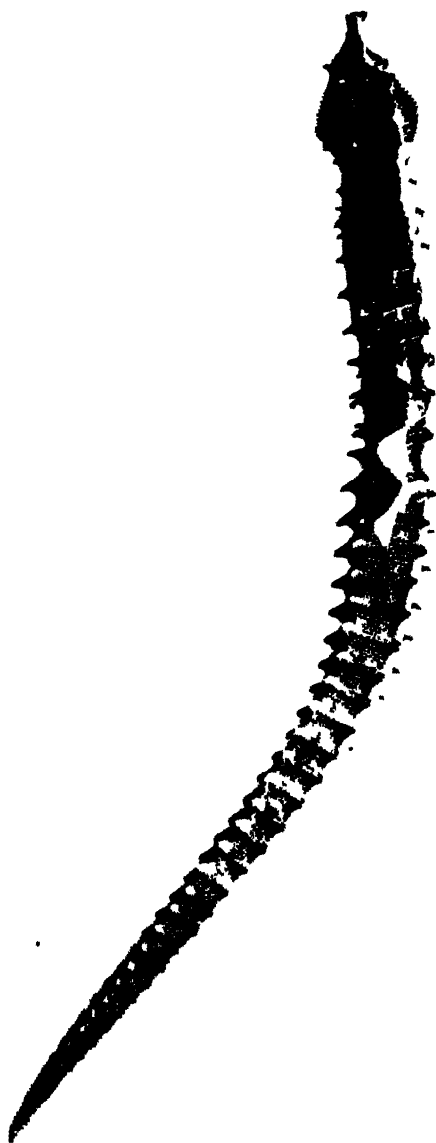
BREDER AND EDGERTON *HIPPOCAMPUS*

PLATE 2.

Back view of *Hippocampus* similar to PLATE 1 showing clearly the amplitude of these waves. The nearly invisible transparent pectorals stand out well from the head.

PLATE 3.

Hippocampus free in water.　Dorsal shows waves of large amplitude as in PLATE 1; pectorals also with large and few waves.　This position is characteristic of faster movement.　In still faster swimming the entire body inclines forward at about the angle shown here for the tail.

BREDER AND EDGERTON· *HIPPOCAMPUS*
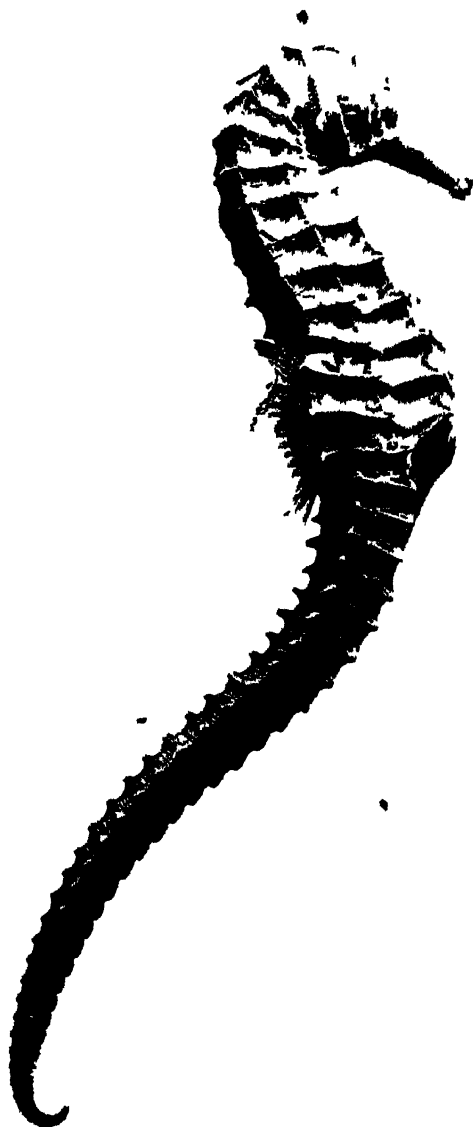
A

B

BREDER AND EDGERTON· *HIPPOCAMPUS*

PLATE 4.

A. Detail of the pectoral of *Hippocampus* free in water. In this position the tail was held coiled and straight down and there were practically no waves in the dorsal. Locomotion was slow, apparently produced by the pectorals alone with waves of intermediate amplitude.

B. Detail of pectoral of *Hippocampus* free in water. In this position the tail was uncoiled and held straight down, and there were waves of an intermediate amplitude in the dorsal fin. Locomotion was moderate, with waves of large amplitude in the pectoral fins.

PROTEIN HORMONES OF THE PITUITARY BODY *

By

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H. L. Fevold, George W. Irving, Jr., C. N. H. Long,
Theodore Shedlovsky, and Abraham White

CONTENTS

INTRODUCTION TO THE CONFERENCE ON
PROTEIN HORMONES
OF THE PITUITARY BODY

By H. B. van Dyke

From the Division of Pharmacology, The Squibb Institute for Medical Research, New Brunswick, N. J.

This gathering of physicists, chemists and biologists has a general or specialized common interest in those fragile giant molecules, the proteins. The curiosities, the languages and the tools of the investigators, however, are otherwise so conspicuously different as to make even Dr. Shedlovsky despair of any attempt to apply "criteria of purity" to the whole group. Perhaps the most important benefit which can come from this conference is the opportunity of learning with what confidence we can speak of the isolation and the physical or chemical characterization of those pituitary hormones which are believed to be proteins. If that purpose is to be accomplished, discussion must be free and frank. In contrast with the limitations imposed at the usual large scientific gathering today, it is fortunately possible not to have to demand serious curtailment of pertinent discussion during the two days at our disposal. The biological aspects of discussion will have to be limited, however, to those phases which must be understood and defined by the investigator interested in proteins extracted from the pituitary. To the profit we can all expect from a complete exchange of data and ideas can be added the pleasure described by Nietzche's epigram, "It is certainly not the least charm of a theory that it is refutable." I hasten to add, however, that conferences like this unfortunately are now scientific luxuries whose number will necessarily be increasingly restricted.

Those members of the conference who are not biologists should be acquainted with the general topography of that complex domain, the biology of the pituitary gland. In an effort to serve only briefly as a guide, I shall have to be dogmatic and speak simply of those confusing phenomena in which biological specialists lose themselves and their readers.

The endocrine or ductless glands are dominated by the pituitary body, distinguished from all the others by its structural intricacy of four distinct parts. Nothing is known of the function of one division, the *pars tuberalis*, which is found as a collar of tissue about the stalk of the pituitary of mammals. This stalk, by which the gland is attached to the base of the brain, is a further distinction of the pituitary in the glandular hierarchy and permits nervous centers, especially of the primitive part

of the brain, to dispatch impulses to a second lobe lying to the back, the neural lobe. A third division, the intermediate lobe, lies between the neural lobe and the fourth part, the anterior pituitary, which, although last on our list, is the largest and most important part.

The physiological dominance of the pituitary among glands of internal secretion depends exclusively upon this largest part, the glandular or anterior lobe. This fact has been demonstrated by all the traditional methods of the endocrinologist. If this lobe has been removed, no other important gland of internal secretion can function to the proper benefit of the whole organism. For example, the thyroid gland, which regulates the pace of heat production in the body, atrophies and secretes only seriously inadequate quantities of its hormone so that the metabolic rate falls and the body temperature is significantly reduced. On the other hand, a suitable anterior pituitary extract will cause the opposite effects if injected: the thyroid hypertrophies; its cells secrete at an abnormally rapid rate; the metabolic rate rises; other signs also resembling overdosage of thyroid extract appear. A similar picture differing in details could be described with respect to the adrenal glands, the ovaries and the testes. The substance or substances regulating growth or metabolism (or both) often act directly, but probably depend upon the presence of one or more endocrine glands, such as the thyroid or adrenals, to exert their full effects. Therefore, the anterior lobe of the pituitary regulates growth, reproduction and metabolism (utilization of food) either directly by acting on non-glandular tissues or indirectly by maintaining or stimulating the important endocrine glands.

For a number of reasons the importance of the anterior pituitary regulation of the gonads (ovaries and testes) has received special attention reflected by intricate hypotheses regarding the controlling mechanisms. The diagram of FIGURE 1 may assist those not familiar with pituitary physiology. At present, the testes appear to be controlled by only two pituitary secretions. Follicle-stimulating hormone maintains the tissue (tubules) in which the male germ cells divide and mature to become spermatozoa, whereas luteinizing hormone stimulates the interstitial cells which secrete the male hormone or androgen necessary for secondary sexual characteristics. Three anterior pituitary secretions appear to direct and operate the complex functions of the ovary. The follicle-stimulating hormone is necessary for complete follicular development culminating in the release of the ovum (ovulation). The remaining cells of the follicle are then transformed into lutein cells (*corpus luteum*) owing to the action of the luteinizing hormone. The latter acts only as a capitalist; it furnishes one of the essential means of erecting the new struc-
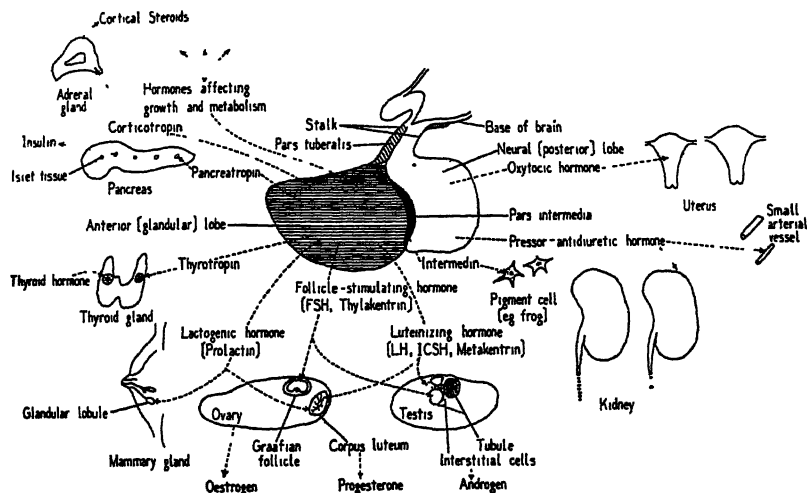
FIGURE 1.—Diagram of pituitary hormone physiology.

ture but contributes nothing to the operation of this luteal body which immediately languishes and has no useful function with respect to pregnancy unless a third pituitary hormone, the lactogenic hormone, is secreted to furnish the managerial or operative stimulus. Thus, lactogenic hormone, so named because its first demonstrated effect was upon the secretion of milk, makes possible the secretion of progesterone which, acting on the prepared uterine mucosa, permits implantation and growth of the fertilized ovum. Ovarian secretion of estrogen and ovulation appear not to occur unless both follicle-stimulating and luteinizing hormones are acting either simultaneously or in close sequence.

Lactogenic hormone likewise supplies the operative stimulus for the secretion of milk but cannot function unless the breasts are prepared for lactation.

Other anterior pituitary hormones are shown in the diagram as stimulating general body growth, affecting metabolism, stimulating the thyroid, the adrenal cortex and the islet tissue of the pancreas. The least striking of these actions is the stimulation of pancreatic islet tissue. The effects on general body growth and particularly those on closely related metabolic processes are ill understood, and, as Dr. Long emphasizes, will not be better comprehended until approximately pure hormones are available for study. Thyroid and adrenal stimulation are peculiarly important because thyroid hormone and the adrenal cortical steroids are so essential for the normal utilization of food and minerals.

Unfortunately, no qualified investigator was able to present a discussion of intermedin, the melanosome-dispersing or chromatophorotropic hormone of the *pars intermedia*. Probably this hormone is a polypeptide. So far as we know the survival of cells secreting intermedin in the mammalian pituitary represents an atavic heirloom since intermedin is principally of interest as a hormone dispersing pigment granules in cells of certain fishes, amphibia and reptiles.

The neural lobe as its name suggests is the only division of the pituitary derived from nervous tissue to which it bears considerable resemblance. The anatomical and secretory integrity of this lobe depends upon an intact connection with the base of the brain by means of the stalk of the pituitary. Extremely active substances causing the contraction of blood vessels, a lessening of the rate of urine formation together with a change in the minerals excreted in the urine, and a contraction of the uterus, can be extracted from neural lobe tissue. The physiological importance of this lobe is most evident with respect to the metabolism of water so far as this is regulated by the kidneys.

The biologist must accept a grave responsibility in performing accurate qualitative and quantitative assays. Here lie fruits of great dissension to which another conference like this could devote its whole proceedings. So far as the general field is concerned, the accuracy, speed and convenience of assay of neural lobe principles have not been excelled. Methods of assaying anterior pituitary hormones are so diverse and are commonly so lacking in accuracy as to represent the biologist's great contribution to much of the existing confusion which can be exorcised only by tolerance, thought, labor and yet more labor. Finally it is well to remember that all qualitative assays (attribution of specific biological effects to extracts) require that the animals used for assay be hypophysectomized. Also, it is increasingly evident that hypophysectomized animals should be chosen for many of the attempts to perform quantitative assays.

In the beginning, I warned you that I should be dogmatic. Although I believe that I have justified that warning, I have frequently been forced to qualify my remarks. Since our knowledge is so imperfect, it should be evident that, so far as events allow, physicists, chemists and biologists must continue to ratify and utilize an alliance which permits all tools of research to be used in the isolation of pure pituitary hormones. Only by this means can we hope ultimately to understand how this extraordinarily complex and dominant gland may ensure health or contribute to disease.

CRITERIA OF PURITY OF PROTEINS

By Theodore Shedlovsky

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INTRODUCTION

A pure chemical substance may be defined in a simple, unambiguous manner as *a quantity of matter consisting of a single molecular species.* However, this definition excludes allotropic, polymeric, isotopic and other "mixtures" which, for many purposes, can usefully be considered to be pure substances.   For example, what is called pure water contains molecules composed of various isotopes of hydrogen and oxygen, probably some polymerized molecules, hydrogen ions, deuterium ions and hydroxyl ions of several isotopic compositions.

The range of the usual molecular weights for proteins is from 20,000 to 200.000 and higher.   This corresponds to large numbers of atoms in each molecule.   Are we to suppose that these atoms have the same spatial arrangement in all the molecules of a pure protein?   Probably not, although we have no experimental way of answering the question. But, since we are not interested in philosophical wrangling, we shall confine our discussion to operational considerations of chemical purity.   Moreover, in this conference on protein hormones we are chiefly interested in knowing whether a particular biological activity is due to most of the substance in a certain preparation or to an impurity in it.   We shall, therefore, lay our emphasis on impurities rather than "purity."

The usual operational criteria for the purity of inorganic and organic materials, which are not megamolecular as are proteins, are constancy of density, refractive index, optical rotation, melting point, boiling point, dielectric constant, electrical conductance, solubility, analytical data, etc., after redistillation, recrystallization, or preparation by different methods.   Unfortunately, most of these operations are not available for proteins.   These are very labile substances, and the procedures which can be used without fear of profoundly altering them are indeed limited.   Also, analytical data on proteins are of relatively little use in most cases for establishing purity, and laboratory synthesis has as yet not been possible.

Among the various physicochemical procedures which are applicable to the study of proteins there are a few which provide the most satisfactory criteria we have for estimating the degree of purity.   These are electrophoretic analysis, observations in the analytical ultracentrifuge,

and the determination of solubility curves in suitable solvents. Proteins form salts with both acids and bases, and, except at the isoelectric point, appear as ions with a net electric charge. Their electrical mobilities depend largely on the pH and on the salt composition of the solution at a fixed temperature. Two different proteins may have identical mobilities in a given solvent, but the probability of the mobilities remaining similar at other values of pH is much smaller. The ultracentrifuge determines sedimentation constants, which depend on the size and shape of the molecules. Here again, two different proteins may happen to have similar sedimentation constants under certain conditions. Determinations of solubility curves involves analogous considerations. However, the likelihood of two different substances behaving alike in all three respects, that is, electrophoresis, sedimentation and solubility, can probably be ruled out in the present state of our knowledge.

TESTS FOR PURITY OF PROTEINS

Before entering on a discussion of various procedures which may be of use in assessing the degree of purity of a certain protein preparation it may be well to classify the following cases: (1) The pure form is available. (2) The pure form is not yet known. (3) The nature of a suspected impurity is known. In the first case the task is simplified by the fact that all the properties of the preparation to be tested can be compared quantitatively and directly with the pure substance. In the second case one is embarking on an uncharted course and the task is correspondingly more difficult. In the third case sensitive tests for the suspected impurity may already be available which can greatly simplify the work required.

In the sections below, various procedures pertinent to our problem will be discussed. The reader is also referred to articles by E. J. Cohn,[1] (physical-chemical characteristics of protein molecules), and by N. W. Pirie,[2] (criteria of purity of large molecules) which deal with the question.

CHEMICAL ANALYSIS, TITRATION CURVES, ETC.

Different proteins are so nearly alike in elementary composition of carbon, oxygen, hydrogen, and nitrogen that assay for these elements cannot tell us whether we are dealing with one protein or several. The determination of sulfur, phosphorus, metals, or of specific groups such as amino acids and carbohydrates, are much more useful, since in many cases they tend to show appreciable differences from protein to protein. Here, much depends on the magnitude of the differences and the accuracy

[1]Cohn, E. J. Chem. Rev. **24**: 203. 1939.
[2]Pirie, N. W. Biol. Rev. Cambridge Philos. Soc. **15**: 377. 1940.

of the analytical methods in relation to the proportions of the proteins in a given mixture.   The usual analytical methods are sufficiently familiar as are the procedures for estimating specific groups.   The estimation of amino acids in a protein from titration curves was discussed by Cannan[3] at a recent conference of the Academy.   Analytical results can give valuable information of a negative nature, *i.e.*, yield values which fail to be reproducible from preparation to preparation of what was thought to be one substance, and they can also be helpful in the estimation of molecular weight.   But if the analytical figures are reproducible, that fact alone provides a poor criterion for purity.

CRYSTALLINITY

Crystallinity of a protein had been considered an indication of purity, but it is now well recognized that it is an unsatisfactory criterion.   To be sure, a homogeneous crystalline preparation assures the absence of substantial amounts of amorphous matter.   Also, it is well known that certain proteins which appear to be quite similar in many respects, such as hemoglobins from several species, have different crystal forms.   However, different crystal forms may be obtained from some single substances, and the crystallinity criterion suffers from other serious limitations.

Crystals which may appear to be homogeneous under a microscope may consist of mixed crystals or solid solutions.   Isomorphism is far from uncommon with proteins.   There is always the possibility of occluded matter being present which cannot be removed readily even after many recrystallizations.   Preparations may seem to be superficially crystalline but actually may not be so.   X-ray analysis can decide this question.   It is, in fact, the only reliable means of ascertaining the degree of internal atomic regularity.   Also, electron microscopy, which is becoming more common, may supply interesting information about structure.

ELECTROPHORESIS

Electrophoresis is concerned with the motion of charged particles by the action of an electric field.   With the moving boundary (Tiselius) method which has become such a powerful tool in the study of proteins and other large molecules, it is possible to obtain very useful information as to the purity of such materials.   It is also possible to effect the separation of components from a mixture, at least in sufficient amounts for many further biological studies.

In principle the method consists in sharply stratifying the solution to be studied below the solvent itself in a U-tube.   With proteins, whose

[3]Cannan, R. K., Kibrick, A., & Palmer, A. H.   Ann. N. Y. Acad. Sci. 41: 243.   1941.

mobilities are sensitive to pH because of their amphoteric nature, buffer solutions against which the preparations are dialyzed serve as solvent. On applying an electric potential all the ions in the tube will move in the field with the result that after a time there will appear boundaries corresponding to the number of components, in addition to those in the solvent, having different mobilities. Also, there will be present the so-called δ and ϵ boundaries which in general move but little if at all. These do not correspond to any components but to buffer salt and total protein gradients left behind in the rising and descending limbs of the tube after the components have moved away from the original boundaries.

The technique which was originally described by Tiselius[4] is now widely used with the improvements of Longworth,[5] who developed the automatic schlieren scanning method, and of Philpot[6] and Svensson,[7] who applied the cylindrical lens and inclined slit method for observing and photographing the boundaries. Thus, electrophoretic patterns are obtained from which the concentrations of the components* having different mobilities can be estimated, and the mobilities can be computed from the volume swept through by the boundaries, the field strength, and the time during which the current had passed.

Electrophoretic patterns obtained with the Longworth[5] method are shown in FIGURES 1, 3 and 4. The patterns of the various fractions arising in the preparation of crystalline ovalbumin at pH 3.92 are shown in FIGURE 1, taken from the classical paper of Longworth, Cannan, and MacInnes.[8] The arrows pointing to the left indicate the rising boundaries and the arrows pointing to the right indicate the corresponding descending boundaries. The δ and ϵ peaks correspond to the boundaries arising from salt and total protein gradients, while the others correspond to various components. The areas under the peaks depend on the component concentrations, while the displacements from the tails of the arrows indicate the relative mobilities. A refers to ovalbumin, G_1, G_2 and G_3 to globulins, C to conalbumin and O to ovomucoid. It is evident that the first albumin fraction (b) (as well as the filtrate) and the globulin fraction (c) are quite impure, containing components recognized in the complete egg white pattern (a). Repeated crystallization (d), (e), (f) results in a product which is quite homogeneous electrically at pH 3.92. But determinations at other values of pH showed that ovalbumin,

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* The optical methods indicate the refractive index gradients in the boundaries. The integrated refractive index gradients correspond to the integrated density gradients which in turn are proportional to the concentrations of the components to which the boundaries owe their origin.

[4]Tiselius, A.   Nova Acta Soc. Sci. Upsala. IV 7 (4).   1930.
[5]Longworth, L. G.   Jour. Am. Chem. Soc. 61: 529.   1939.
[6]Philpot, J. S. L.   Nature 141: 283.   1938.
[7]Svensson, H.   Kolloid-Z. 87: 181.   1939; 90: 141.   1940.
[8]Longworth, L. G., Cannan, R. K., & MacInnes, D. A.   Jour. Am. Chem. Soc. 62: 2580. 1940.
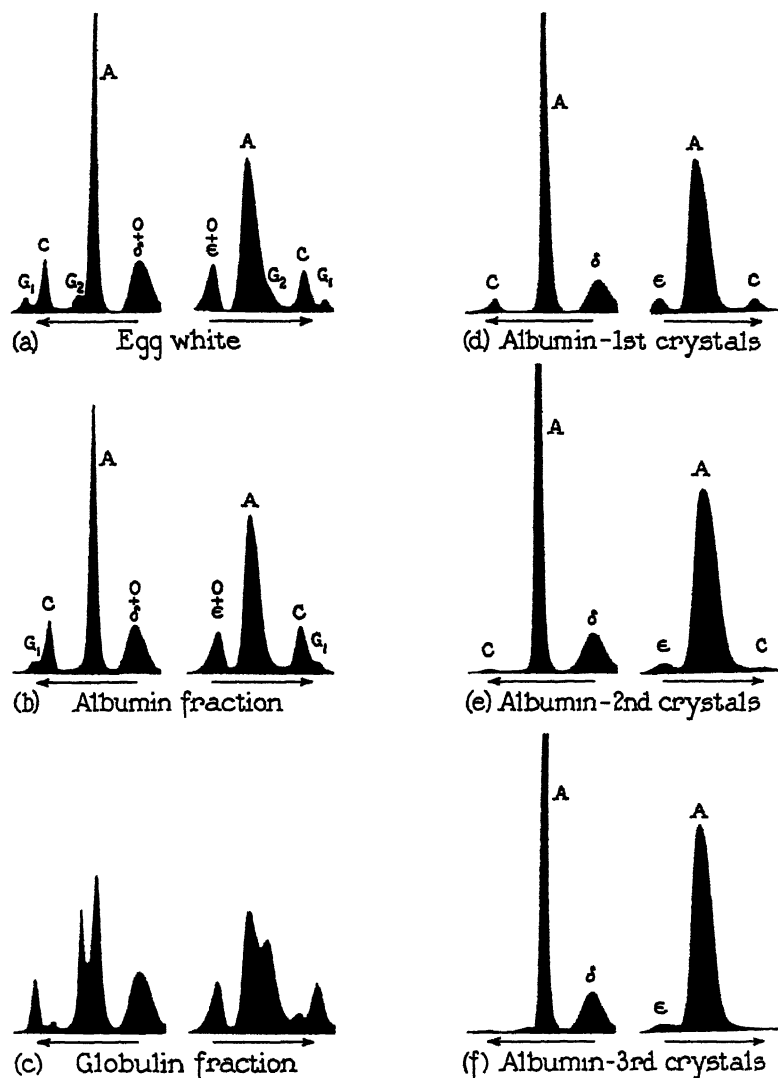
FIGURE 1. Electrophoretic patterns of egg white and its various fractions at pH 8.92 [from Longsworth, Cannan and MacInnes[8]].

A, and conalbumin, *C*, are complex. The mobility curves, as a function of pH, for the components of egg white are shown in FIGURE 2.
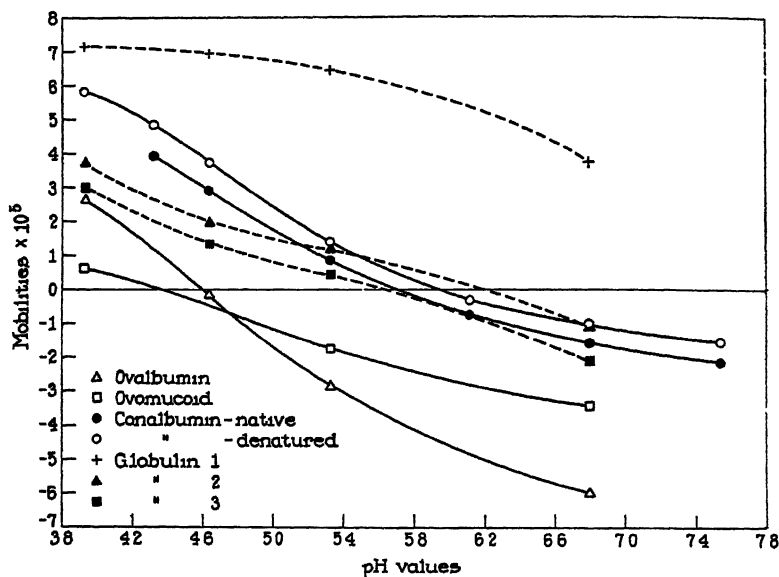
FIGURE 2.   Mobility pH-curves for the components of egg white [from Longsworth, Cannan and MacInnes[3]].

The reason for the absence of two conalbumins in FIGURE 1 is due to the fact that a transition takes place between the two forms which is a function of pH.   Below pH 4 only one form predominates.   The iso-electric points are at the values of pH corresponding to zero mobility.

It is evident that electrophoretic analysis must be carried out at different values of pH to provide as much information as possible regarding purity.   Mobility–pH curves in conjunction with relative concentration determinations obtained from the patterns supply the maximum information.   It should be noted here that variation of salt concentration (ionic strength) tends to displace the mobility curves.   A tenfold change in ionic strength tends to alter the mobility of proteins by an amount corresponding roughly to the effect of one pH unit.[9]

In FIGURE 3(a) a pattern of the rising boundary from a nucleoprotein preparation is shown.   The small peaks correspond to soluble impurities, and the opaque region to impurity in the form of a small quantity of suspended matter.   With sufficient care the electrophoretic method is capable of detecting a few tenths of a per cent of an impurity which has

[9]Davis, B. D., & Cohn, E. J.   Jour. Am. Chem. Soc. 61: 2092.   1939.
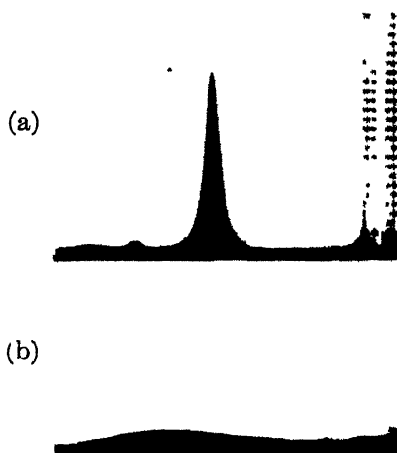
FIGURE 3. (a) Electrophoretic pattern of a nucleoprotein preparation. Rising boundary. (b) Electrophoretic pattern of a protein which had been badly degraded. Rising boundary.

a mobility different from the major substance. FIGURE 4 shows a pattern obtained with the cylindrical lens method[6,7] from a protein preparation which evidently contains at least three components.

Occasionally, one observes considerable electrical heterogeneity of a different sort. The pattern shown in FIGURE 3(b) was obtained from a preparation of protein which had been badly maltreated. It is evident that no sharp boundary exists. The material apparently had been broken up into fragments consisting of particles having a very wide range of mobility.
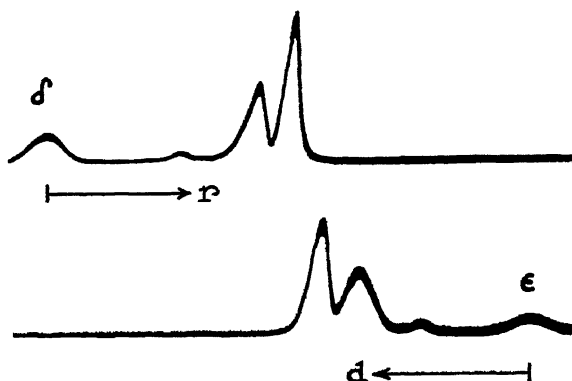
FIGURE 4. Electrophoretic pattern obtained with cylindrical lens of a protein preparation containing three components. Rising boundary (upper curve), descending boundary (lower curve).

Electrophoresis has been very valuable in supplying an excellent cri-
terion of purity for proteins, although it, alone, is not sufficient, since it
tells only whether the material is electrically homogeneous or not. As
an example of its use in purification, I cite the work of Seibert[10] who used
electrophoresis successfully both as a preparative method and as a guiding
assay for finding suitable precipitating conditions to purify tuberculin
protein contaminated with nucleic acid and polysaccharide. Quoting
from her summary: "Nucleic acid and polysaccharide could not be re-
moved quantitatively from the protein by isoelectric precipitation, by
electrodialysis, by dialysis at pH 2.1, by repeated precipitation for half
saturated ammonium sulfate at the isoelectric point, or by repeated pre-
cipitation with 2 or 10% trichloracetic acid. Electrophoretic mobility
curves revealed the fact that on the acid side of pH 5 the nucleic acid and
the protein migrated as one component, whereas at less acid reactions
the two travelled with very different mobilities. The polysaccharide
was immobile at all pH values. Therefore by means of repeated electro-
phoresis at pH 7.3 it was possible to remove both nucleic acid and poly-
saccharide from the protein with no loss of potency. It was, further-
more, possible to remove both of these impurities from the protein by
repeated precipitation on the alkaline side of pH 5, *e.g.*, by half saturated
ammonium sulfate at pH 7, with no loss of potency." A detailed elec-
trophoretic study of complex formation between yeast nucleic acid and
ovalbumin with a theoretical analysis of the results was reported recently
in an important paper by Longsworth and MacInnes.[11]

CENTRIFUGAL SEDIMENTATION

The ultracentrifuge first developed by Svedberg[12] provides a very val-
uable method for determining the molecular weights of large molecules.
Sedimentation is achieved by the use of high speeds which result in suffi-
ciently high centrifugal fields. In most work with proteins, fields of
about 200,000 times gravity are produced in rotors spinning at about
60,000 r.p.m. I shall not discuss any of the experimental details beyond
stating that the optical systems usually employed at present for observing
the sedimentation boundaries resemble those used in electrophoretic work.
A detailed treatise on the technique appears in Svedberg and Pedersen's
book,[12] and an interesting monograph on the subject has been published
recently by the Academy.[13]

[10] Seibert, F. B.   Jour. Biol. Chem. 133: 593.   1940.
[11] Longsworth, L. G., & MacInnes, D. A.   Jour. Gen. Physiol. 25: 507. 1941–42.
[12] Svedberg, T., & Pedersen, K. O.   "The Ultracentrifuge." Clarendon Press. Oxford.
[13] "The Ultracentrifuge." Ann. N. Y. Acad. Sci. 43: 173–252. 1942.

The sedimentation velocity of particles depends on their density, size, and shape in accordance with the following equations:

$$s = \frac{D(1 - \bar{v}\rho)M}{RT}$$

$$D(f\,f_0) = \frac{RT}{6\pi N\eta} \left(\frac{4\pi N}{3vM}\right)^{1/3}, \tag{2}$$

in which the symbols have the following significance:

    M—molecular weight
    s—sedimentation constant
    D—diffusion constant
    T—absolute temperature
    R—gas constant
    N—Avogadro's number
    $\bar{v}$—partial specific volume
    ρ—density of the solvent
    η—viscosity
  $f\,f_0$—the shape factor, which is unity for spherical molecules.

If the sedimenting molecules are spherical, the molecular weight can be obtained from the centrifugal data alone, by equation (1), since $f\,f_0$ is unity and D is given by equation (2). Otherwise D must be determined. For obtaining molecular weights it is best to work at a pH near the isoelectric point of the protein. Measurements on diffusion make possible the calculation of the shape factor, f/f_0, from equation (2). Or, conversely, it is possible to determine the degree of asymmetry in the molecular shape if the molecular weight is known. However, if the shape is very elongated, the sedimentation velocity will not be very sensitive to length.

The limitations of the ultracentrifuge as an instrument for providing a criterion of purity are that similar sedimentation constants only indicate similar molecular weights of molecules having the same shape or of compensating values of M and $f\,f_0$. Also, there is a certain dependence on concentration, the sedimentation generally being faster in more dilute solutions. This fact accounts, at times, for spurious sharpness of boundaries; a small amount of slightly smaller material, being in a region of low concentration, will tend to catch up to the leading boundary, thus disturbing the resolving power. However, under favorable conditions, it is possible to detect impurities having sufficiently different sedimentation constants from the major component, if they comprise 2 or 3 per cent of the material.
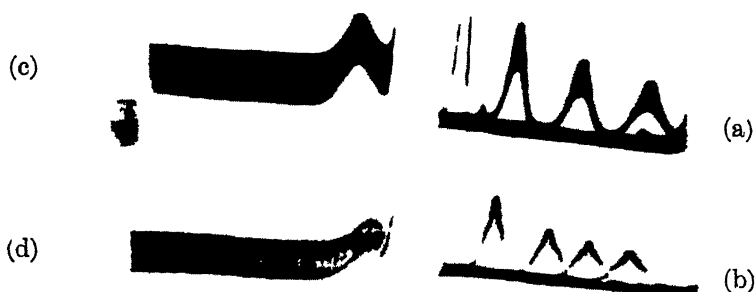
FIGURE 5. Sedimentation patterns. Metakentrin preparations, (a) and (b); Horse globulin preparations, (c) and (d) [Rothen].

The patterns shown in FIGURE 5 were supplied by Dr. A. Rothen. The two on the right are from preparation of hog metakentrin, photographic exposures having been made at intervals as the sedimentation proceeded. A small quantity of a more rapid component (two small peaks) will be noted in the upper patterns, (a). This is absent from another preparation shown in the patterns directly below, (b). On the left of FIGURE 5, centrifuge patterns, (c) and (d) (single exposures), or horse globulin preparations are shown.

As an example of nonhomogeneous material, patterns obtained from urea-denatured ovalbumin (Rothen) are shown in FIGURE 6, which contains several exposures taken at intervals of time during the sedimentation. It should be pointed out, however, that materials which lack homogeneity in the centrifuge need not necessarily also do so in electro-

FIGURE 6. Sedimentation patterns of nonhomogeneous material. Urea denatured ovalbumin [Rothen].

phoresis (since mobility depends primarily on charge distribution and not on size and mass), and vice versa.

SOLUBILITY

Theoretically, solubility provides one of the best criteria for the purity of a substance, since it is based on sound thermodynamic principles. It involves the measurement of a partition equilibrium of a substance distributed between two phases. In the case of protein solubility the distribution is between the solid itself (the solid phase) and a solution of it, usually in an aqueous buffered salt medium (the liquid phase). The basic theory which gives quantitative expression for such systems is summarized in the Phase Rule of Willard Gibbs. At equilibrium, the chemical potential of any neutral species is the same in all phases. The chemical potential of a substance in any phase depends on its mol fraction in that phase and on its corresponding activity coefficient which is a measure of deviation from Raoult's law, or "ideal" behavior. A substance will continue to dissolve in a solvent until its chemical potential in the solution builds up to just the value it has in the solid, provided there is sufficient solid available. If the solid is homogeneous (one solid phase) but contains the substance in impure form (solid solution), the chemical potential of the substance is less than it would have been in the pure state because its mol fraction is unity when pure and less than unity when the solid phase contains other components. At equilibrium, the composition of the solid solution is no longer what it was originally, unless the relative solubilities of the components happen to be of just the right ratio. If this should happen to be so, changing the solvent will greatly minimize the likelihood of such compensation. Therefore, it is desirable to make solubility determinations in more than one solvent to derive all the advantages of the solubility method for establishing purity. The addition of more of the original material (solid solution) will again disturb the composition of the solid phase so that, in general, more material will be dissolved. Sorensen,[14] who was the first to apply the solubility method in studying proteins, found that proteins which had been considered pure were actually not so since their solubility increased with the quantity of solid used in the determinations with a given volume of solvent. The method, which has been developed admirably by Northrop and his collaborators[15,16] depends on the determination of the quantity of protein appearing in a constant volume of buffered solvent, at constant temperature and pressure, as the total quantity of

[14]Sorensen, S. P. L.   Compt-rend. trav. Lab. Carlsberg **15**: 1.   1925; **18**: 1.   1930.
[15]Northrop, J. H., & Kunitz, M.   Jour. Gen. Physiol. **13**: 781.   1929–30.
[16]Kunitz, M., & Northrop, J. H.   Cold Spring Harbor Symposia **6**: 325.   1938

protein is increased. It is best to use solvents which dissolve but little of the material. The concentration of protein, which is proportional to its mol fraction in dilute solutions, is usually determined through nitrogen analyses. The accuracy of the analytical method is one of the important limitations in the technique.

On preparing a plot of total protein against protein in solution, one should obtain, for a pure substance, points which fall on two straight lines. As long as all the material added goes into solution, the points should naturally fall on a straight line with unit slope. When saturation has been achieved no more material should dissolve on further addition of substance, and the subsequent points should therefore fall on a horizontal line, producing a sharp break with the line of unit slope. It is important to get as many points as possible in the vicinity of the break to make sure of its reality.

If the preparation consists of several substances present as a physical mixture, but not in solid solution, we are dealing with several solid phases. Here we will observe several breaks in the plot before it becomes horizontal. Such a plot, for three pure solid phases, is shown in FIGURE 7, taken from Kunitz and Northrop's paper.[16] At point B the solution is saturated with substance 1, at point C it is also saturated with substance 2, and at D it has become saturated with all three. The plot must be horizontal beyond D and must have unit slope from A to B. On extrapolating the lines BC, CD, and DE to the ordinate axis, the intercepts show the solubilities $S1$, $S1 + S2$, and $S1 + S2 + S3$, respectively. In FIGURE 8, also from Kunitz and Northrop,[16] solubility curves for α and γ chymotrypsin mixtures, which do not form solid solutions, are shown.

Total solid added
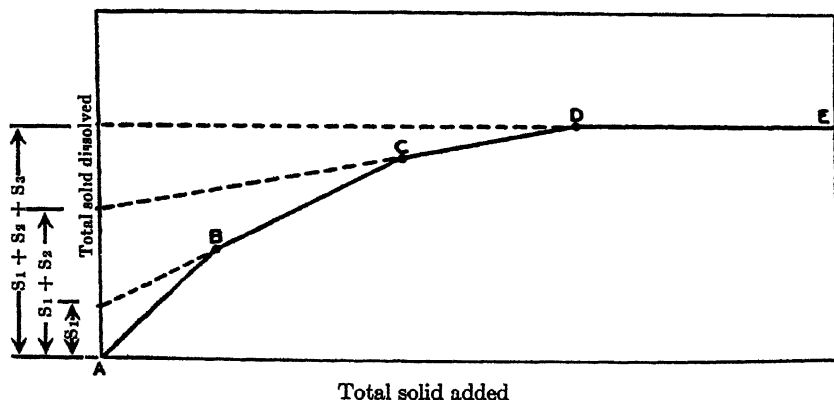
FIGURE 7. Theoretical solubility curve for three pure solid phases.
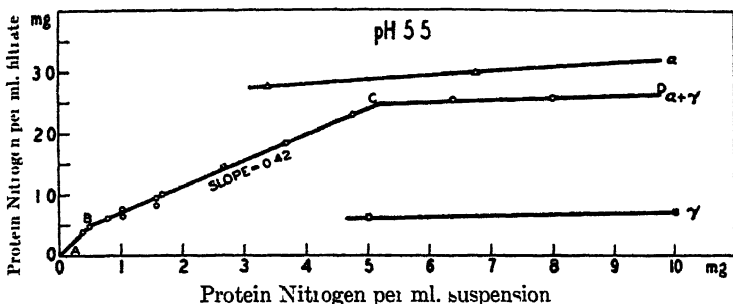
FIGURE 8   Solubility of artificial mixture of crystals of α and γ chymotrypsin 40 per cent α + 60 per cent γ in 0 4 saturated ammonium sulfate pH 3 5 at 10° C. Slope BC measured = 0.42, calculated = 0 48. [Kunitz and Northrop[10].]

The uppermost curve, α, and the lowest curve, γ, indicate the solubilities of α and γ chymotrypsin by themselves, from which the slope BC in the middle curve can be obtained if one knows the proportions of α and γ in the mixture, or vice versa.   It will be noted that the α chymotrypsin curve slopes gently instead of being horizontal.   This indicates a small amount of impurity present in solid solution.

For solid solutions, one does not obtain linear plots with breaks, but curves which show early deviations below the initial unit slope, and which continue to rise as the amount of total solid added is increased.   If such curves are found one may be sure that the preparation is impure.   On the other hand, if a unit slope followed by a horizontal line is obtained, it is an excellent criterion of purity, especially if the same result holds in other solvents.   In conjunction with electrophoretic and ultracentrifugal homogeneity, this provides the most satisfactory evidence in our possession for the purity of proteins.

BIOLOGICAL ACTIVITY

Specific biological activity itself must tell us whether it is the almost pure substance or the impurity in which we are really interested.   It must be borne in mind that different proteins, from different species, may have similar biological activity (isobiological), although they differ physicochemically and serologically, and can thus be distinguished.   A protein molecule may also be capable of undergoing more or less profound alteration which may decrease, increase, destroy or retain a biological activity originally present.   From this standpoint great care is necessary in achieving purification lest "the stream be found purer at its source."

Constant biological activity must be considered, from the practical point of view, as a most important criterion of purity of biologically

active pioteins if sufficiently accurate methods of assay are available. The activity may depend upon a small portion of the molecule, the prosthetic group, such as iron in hemoglobin, copper in hemocyanin, thyroxin in thyroglobulin. riboflavin in Warburg's yellow enzyme, etc. The same prosthetic group may be a component part of otherwise different proteins which therefore exhibit similar biological activity. Such substances, isobiological in one respect may be heterobiolological in others. For example, hog and sheep metakentrin both possess similar biological activity as hormones but can be distinguished from one another by serological means. Also, they differ physicochemically as proteins, having, among other things, different isoelectric points. However, in many proteins, such as insulin, certain enzymes, etc., no prosthetic groups have been identified with their specific biological behavior.

DISCUSSION AND SUMMARY

The judgment of purity of a protein or other substance is a practical matter. Where biologically active substances are concerned the evidence required for reaching a reasonable judgment must come from the biologist, the organic chemist, and the physical chemist. Purity should then be defined in operational terms, the more evidence the better. We should have accurate biological assays from the biologist, if possible; evidence from the chemist regarding constancy of analytical figures for certain specifically significant groups or atoms; evidence from the physical chemist regarding homogeneity in electrophoresis. the ultracentrifuge, and solubility. These factors have been discussed briefly and the principles on which the physicochemical methods are based have been outlined.

If the biologist and physical chemist can agree as to what fraction of a preparation appears to be "the substance" and what appears to be impurity, then the evidence from the physical chemist provides the best criteria of purity within the limitations of the experimental methods.

HORMONES OF THE POSTERIOR LOBE OF THE PITUITARY GLAND

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INTRODUCTION

In the fifty years that have elapsed since the first demonstration of the blood pressure raising action of injected extracts of the posterior hypophysis[1, 2] a remarkable number of biological properties have been attributed to this gland. Besides exhibiting a pressor effect, an aqueous extract of the posterior lobe may be capable of effecting contraction of the musculature of the uterus, intestine, stomach, esophagus and gall bladder; of influencing the secretion of urine, milk, gastric and pancreatic juice and bile, and of exerting various effects upon the heart, respiration, metabolism and pigmentation. It is possible that several of these effects may be due to the presence in crude extracts of substances, like histamine, which are not peculiar to the posterior lobe but extractable from a variety of other animal tissues. Moreover, a few of the actions mentioned may possibly be due to substances which arise in portions of the hypophysis other than the posterior lobe, present as contaminants in posterior lobe extracts. Certain of these effects have been sufficiently well characterized, however, to justify their classification as pharmacological, if not truly hormonal, properties of the posterior lobe itself. The most clearly established pharmacological properties of posterior lobe extracts are the effect upon mammalian blood pressure (pressor action), the effect upon uterine muscle (oxytocic action), and the effect upon the rate of urine excretion (antidiuretic action). It is with these three activities and particularly the pressor and oxytocic activities, that we are concerned in this discussion.

Thus far, the use of the singular or plural of the word hormone has been avoided in referring to the properties of the posterior lobe since at present it is impossible to decide whether only one or more than one active principle exists in the gland under physiological conditions. Furthermore, in the biochemical approaches to the posterior lobe problem, as in most investigations of the endocrines, the gland tissue is subjected to conditions which are far from physiological. Consequently, a dis-

[1] Oliver, G., & Schäfer, E. A. Jour. Physiol. 18: 277. 1895.
[2] Howell, W. H. Jour. Exp. Med. 3: 245. 1898.

tinction must be made between the chemical conditions that prevail in the living gland and those that exist in the dead tissue extracts commonly employed in laboratory investigations. As practically all of the biochemical data to be discussed have been obtained with gland extracts, it may be advisable to make a brief preliminary examination of the evidence to determine, if possible, whether we may consider the several activities of posterior lobe extracts to be the properties of a single molecule or of separate molecules.

No pure crystalline compounds possessing any of the three activities mentioned have been isolated from posterior lobe tissue. However, by fractionation procedures Kamm,[3] Stehle,[4] and du Vigneaud[5] and their respective associates, have obtained non-crystalline preparations of high pressor potency and very low oxytocic activity. Similarly, amorphous preparations of high oxytocic potency and negligible pressor activity have been obtained. Such fractionations can be carried out without destruction or loss of either activity and no qualitative differences can be detected between the pharmacological responses obtained with each of the separate fractions and the corresponding effects exhibited by unfractionated extracts. Additional evidence which favors the separability of the pressor and oxytocic activities can be found in the work of Dudley,[6] Schlapp[7] and Draper[8] who found that butyl alcohol extraction of acid posterior lobe extracts removed oxytocic activity at a much greater rate than it removed the pressor activity. Electrophoresis experiments[9][10] have shown that the mobility of the pressor activity is many times greater than the oxytocic, both in solutions of purified fractions and in the untreated press juice from fresh posterior lobes. Finally, it has been shown that the substances responsible for the pressor and oxytocic activities possess different isoelectric points.[11]

From these facts it would appear that by suitable treatment of posterior lobe extracts a substance which accounts for the pressor activity can be separated from one which accounts for the oxytocic activity and that each fraction can exert its pharmacological effect independently of the other. Considered in this light it is justifiable to speak of the substances responsible for these actions as the pressor and oxytocic principles or hormones.

[3] Kamm, O., Aldrich, T. B., Grote, I. W., Rowe, L. W., & Bugbee, E. P. Jour. Am. Chem. Soc. **50**: 573. 1928.
[4] Stehle, R. L.,& Fraser, A. M. Jour. Pharmacol. **55**: 136. 1935.
[5] Irving, G. W., Jr., Dyer, H. M., & du Vigneaud, V. Jour. Am. Chem. Soc. **63**: 503. 1941.
[6] Dudley, H. W. Jour. Pharmacol. **14**: 293. 1919.
[7] Schlapp, W. Quart. Jour. Exp. Physiol. **15**: 327 1925.
[8] Draper, W. B. Am. Jour. Physiol. **80**: 90. 1927.
[9] du Vigneaud, V., Irving, G. W., Jr., Dyer, H. M., & Sealock, R. R. Jour. Biol. Chem. **123**: 45. 1938.
[10] Irving, G. W., Jr., & du Vigneaud, V. Jour. Biol. Chem. **123**: 485. 1938.
[11] Cohn, M., Irving, G. W., Jr., & du Vigneaud, V. Jour. Biol. Chem. **137**: 635. 1941.

Evidence concerning the antidiuretic effect is far from convincing but there is a possibility that this effect may also be due to a separate principle. Kamm[3] found that antidiuretic activity was extremely low in his purified oxytocic fractions but was present in large amounts in potent pressor preparations. This indicated that the oxytocic principle was probably not concerned with antidiuretic activity. Moreover, Gilman and Goodman[12] claim that antidiuretic activity is resistant to the action of certain reducing agents which destroy oxytocic activity. Heller[13] has shown that during heat inactivation over a wide pH range (0.57 to 10.0), pressor activity is lost slightly more rapidly than antidiuretic activity, indicating that these two effects may be due to different agents. Until more data are available, conclusions regarding the status of the substance responsible for antidiuretic activity must be withheld.

On the basis of the evidence outlined above, workers have come to regard the pressor and oxytocic activities as manifestations of two separate hormones. It should be emphasized, however, that none of this evidence precludes the possibility that the separate hormones may be linked together in the form of a single, composite molecule, in the gland itself or even in aqueous gland extracts. The proof of the existence of such a "mother-molecule" rests upon its isolation and the demonstration that it not only accounts for all of the pharmacological properties of the gland, but also lends itself to fragmentation under the relatively mild conditions employed for the fractional separation of the pressor and oxytocic principles. Professor Abel and his collaborators at Johns Hopkins long maintained that the activities of the posterior lobe were properties of a large, labile "mother-molecule" but they were unable to secure conclusive proof.[11] In recent months, however, van Dyke and his associates at the Squibb Institute have gone a long way toward achieving this goal.[15] These investigators have isolated from the posterior lobe of oxen a protein which appears to be homogeneous and which possesses pressor, oxytocic and antidiuretic activity. Ultracentrifugal data obtained by Rosenfeld[16] also indicate that the pressor and oxytocic activities in the press juice from fresh posterior lobes may be associated with a rapidly sedimenting substance, presumably a protein, which is not present in purified preparations of the two principles.

From this brief survey it is clear that it is possible to obtain from extracts of the posterior lobe separate chemical entities which are respon-

[12] Gilman, A., & Goodman, L. Jour. Physiol. **90:** 113. 1937.
[13] Heller, H. Jour. Physiol. **96:** 337. 1939.
[14] Abel, J. J. Jour. Pharmacol. **40:** 139. 1930.
[15] van Dyke, H. B., Chow, B. F., Greep, R. O. & Rothen, A. Am. Jour. Physiol. **133:** 473. 1941; Abstracts, 102nd Meeting Am. Chem. Soc. 1941, p. B-10.
[16] Rosenfeld, M. Bull. Johns Hopkins Hosp. **66:** 398. 1940.

sible for the oxytocic and pressor activities, respectively; but it is not clear whether or not these separate entities exist as such in the gland and in certain types of extracts made from the gland. As we have pointed out, recent work makes it necessary seriously to consider anew the possibility of the existence of a "mother-molecule" which possesses both activities, and which is cleaved in the course of extraction or fractionation. We shall consider this point in greater detail later in this report.

In discussing the chemistry of the posterior lobe we are concerned with a review of the properties and behavior of the several active, amorphous preparations that have been isolated from the gland by various procedures. First we shall discuss very briefly the assay techniques used in these investigations. Next we shall discuss the methods of preparation and the chemical and physical properties of the separate pressor and oxytocic preparations. Finally, we shall make a similar survey for the protein isolated by van Dyke and his associates. With this information before us we may be in a position to make further comment upon the unitary and multiple hormone concepts.

ASSAY METHODS

Pressor Assay

The development of the pressor assay method is largely due to the work of Hamilton and Rowe.[17-19] The test animal is the anesthetized dog or cat. Changes in blood pressure in a cannulated carotid artery are registered by means of a mercury manometer and recorded on a smoked drum of the familiar kymograph. The solution to be assayed is injected into a leg vein and, after an interval of 15 minutes, is followed by an injection of the reference standard. Alternate injections of the unknown, in increasing amounts, and the standard are made at 15 minute intervals (to avoid tachyphylaxis) until the blood pressure rise due to the unknown is approximately equal to that produced by the standard in at least two successive pairs of responses. Since the pressor content of the standard dose is known, the ratio between the heights of the standard and unknown responses gives directly the pressor content of the volume of unknown injected. As used routinely, the accuracy of the method is hardly better than ± 20 per cent.

The pressor standard used is a powdered preparation of desiccated fresh beef posterior lobes (Standard Powder) which contains 2 International Pressor Units per mg. when prepared as prescribed by the U. S.

17 Hamilton, H. C.   Jour. Am. Pharm. Assoc. 1: 1117.   1912.
18 Hamilton, H. C., & Rowe, L. W.   Jour. Lab. Clin. Med. 2: 120.   1916.
19 Rowe, L. W.   Endocrinology 13: 205.   1929.

Pharmacopoeia. This standard was originally established by the Conference on Biological Standardization of the League of Nations to serve as the oxytocic standard[20] but has since been adopted as the standard for pressor and antidiuretic assay.[21] It is agreed that 1 mg. of the Standard Powder contains 2 units of each of the three activities.

The pressor assay dose varies between 0.1 and 0.5 unit (0.05 to 0.25 mg. of Standard Powder). Since this small amount of material usually produces a blood pressure rise of approximately 10 mm. of mercury, the pharmacological effectiveness of the pressor principle is apparent. This effectiveness is better appreciated when it is realized that only 0.5 to 2.5 micrograms of a purified pressor preparation (200 units per mg.) is required to produce the same response.

Oxytocic Assay

Two methods are available for oxytocic assay. One of these involves the measurement of contraction in an isolated strip of uterus of the guinea pig. The second method involves the measurement of the fall in blood pressure in the fowl.

The uterine strip procedure was suggested and developed by Dale and Laidlaw[22] and was later modified by Burn and Dale.[23] The official method of the U. S. Pharmacopoeia X is essentially a refinement of their original technique.

One horn of a virgin guinea pig uterus is suspended in a constant 37° C. bath of a balanced physiological salt solution (usually Ringer-Locke solution). One end of the strip is fixed while the free end is fastened to a light lever which registers the extent of movement on a kymograph drum. The solution to be assayed and the standard are added to the bath alternately in varying doses until quantities of the two solutions are found which give equal, sub-maximal contractions in at least two successive pairs of responses. After each contraction the bath solution is discarded and renewed. The oxytocic activity of the unknown is calculated from the ratio between the unknown and standard responses. The accuracy is ± 20 per cent.

The depressor action of pituitary extracts upon the blood pressure of decapitated ducks was first observed by Paton and Watson.[24] Subsequent investigation has shown that the effect can also be produced in anesthetized fowls and that it is due to the oxytocic principle. This

[20] **League of Nations,** Report by the Second International Conference on Biological Standardization of Certain Remedies. Geneva. August, 1925. p. 14.
[21] **League of Nations.** Quart Bull. of the Health Organization **5:** 372, 582. 1936.
[22] **Dale, H. H., & Laidlaw, P. P.** Jour. Pharmacol. **4:** 75. 1912.
[23] **Burn, J. H., & Dale, H. H.** Med. Res. Counc. Spec. Rep. Series **No. 69.** 1922.
[24] **Paton, D. N., & Watson, A.** Jour. Physiol. **44:** 413. 1912.

effect has recently been utilized by Coon,[25] working in the laboratory of Professor Geiling at the University of Chicago, in developing the fowl blood pressure method for oxytocic assay.

Blood pressure changes in a cannulated leg artery (ischiatic) of an anesthetized hen or rooster are recorded in a manner similar to that used in the pressor assay. The solution to be assayed is injected into an exposed leg vein (crural) and it is followed, after a 3 to 5 minute interval, by an injection of the reference standard. Alternate injections of standard and unknown are made in this manner until closely similar depressor responses are obtained. The oxytocic activity of the injected dose of unknown is then calculated from the ratio between these two responses. Reproducibility and accuracy are as good with the fowl blood pressure method as with the uterine strip technique and the former offers the advantage of being a less temperamental test subject. The two methods agree well in the assay of unknowns provided the pressor: oxytocic ratio does not exceed 2.5. However, Coon argues that there is no reason to suspect the fowl blood pressure values as being the incorrect ones in such cases.

The oxytocic standard is the previously described Standard Powder containing 2 oxytocic units per mg. In the uterine strip method a dose of 0.2 unit produces a contraction of several mm. In the fowl a similar dose (0.4 to 0.8 micrograms of a purified oxytocic preparation) produces a blood pressure drop of 20 to 40 mm. The enormous pharmacological effectiveness of the oxytocic principle is obvious.

Antidiuretic Assay

The usual method for antidiuretic assay is similar to that of Gibbs[26] as modified by Burn.[27] Five·cc. of water per 100 gm. body weight are administered by stomach tube or intraperitoneal injection to each of several rats and the solution to be assayed is injected into each animal. The rats are caged together and the time required for the rate of urine excretion to reach a maximum, or the time required for the excretion to attain 50 per cent, is determined. Another group of rats is treated similarly except that a known amount of standard is injected instead of the unknown. The ratio between the standard and unknown time intervals serves as the basis for calculating the antidiuretic activity of the unknown. The error is usually less than 20 per cent if precautions are taken to rule out the individual variations between groups of test animals.

The assay dose is approximately 0.006 unit per 100 gm. body weight.

[25] **Coon, J. M.**   Arch. inter. Pharmacodynamie **62**: 79.   1939.
[26] **Gibbs, O. S.**   Jour. Pharmacol. **40**: 129.   1930.
[27] **Burn, J. H.**   Quart. Jour. Pharm. **IV**: 517.   1931.

This dose reduces the rate of urine excretion to about 50 per cent of that found in an untreated animal. Since the method is capable of differentiating doses differing by as little as 0.002 unit (approximately 1 microgram of Standard Powder), the weight of purified fractions required to produce a similar response is vanishingly small.

PURIFICATION OF THE PRESSOR AND OXYTOCIC PRINCIPLES

Procedures for the purification of the active principles have depended, in general, upon the solubility of the principles in water or aqueous solvents and their relative insolubility in organic solvents and in concentrated salt solutions. However, the principles are soluble to some extent in the lower alcohols, particularly if small amounts of water are present, and this solubility increases markedly as the principles are purified. In some cases solubility in organic solvents is also increased by the presence of acid. In partially purified preparations, the oxytocic principle is more soluble than the pressor in certain organic solvents. Use has been made of this property in procedures for separating these two principles. The principles are appreciably soluble in a few of the lower molecular weight fatty acids, acetic acid being the best of this series. Various organic acids and metallic salts as well as numerous adsorbents have been used with varying degrees of success for the purification and separation of the principles. Unfortunately, separation of the active material and the reagent, or elution of the active material from the adsorbent, usually results in sufficient loss or destruction of activity to discourage extensive use of such procedures. Recently, however, Potts and Gallagher[25] have used chromatographic adsorption on artificial zeolites as a means for separation and purification of the pressor and oxytocic principles. No details have been published but it appears that separate pressor and oxytocic preparations having potencies much higher than any previously reported have been obtained by this procedure.

For the initial gland extract dilute acetic acid (0.05 to 0.5 per cent) has been favored since it is a good solvent and since the pH of the resulting solution lies close to the pH of maximum stability (pH 3.0).

Three methods for obtaining separate pressor and oxytocic preparations, each adaptable to large scale work, will be described to illustrate the steps involved, the yields obtained and the potencies of the products. The steps involved in these three procedures are shown schematically in TABLES 1–3. For comparative purposes the yields given have been

25 Potts, A. M., & Gallagher, T. F. Proc. Soc. Biol. Chem., Jour. Biol. Chem. **140**: p. ciii. 1941. Jour. Biol. Chem. **143**: 561. 1942.

recalculated to show the amounts of purified pressor and oxytocic fractions which can be obtained from 1 kg. of dry posterior lobes or its equivalent in fresh gland tissue. Potencies of products are expressed in terms

TABLE 1
PURIFICATION PROCEDURE OF KAMM AND COWORKERS[3]

Fraction	Weight	Procedure	Potency, Units per mg.		Total activity	
			Pressor	Oxytocic	Pressor	Oxytocic
	gm.				Per cent	Per cent
A	1000	Acetone desiccated posterior lobes Extracted with hot 0.25 per cent HAc; concentrated; saturated with $(NH_4)_2SO_4$. ↓	1	1	100	100
B		Precipitate, dried Extracted with glacial HAc; ether and petroleum ether added to extract. ↓				
C	68	Precipitate Dissolved in 98 per cent HAc; ether added; repeated once.	11	12	75	82
D	63	Precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether to give several precipitates. ↓	10	7	63	44
E	5.9	Most potent precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether as above. ↓	62	14	37	8.3
F (Pressor)	2.0	Most potent precipitate ↓ HAc-ether mother-liquor treated with trace of water and excess of petroleum ether. ↓	80	15	16	3.0
G (Oxytocic)	2.4	Gummy precipitate	6	160	1.5	38

of International Units per mg. of solid. It should be noted that as these figures represent averages in most cases, the yields and potencies of the products obtained in individual experiments will vary slightly from those given.

The figures in TABLES 1-3 indicate that the most potent pressor preparations contain approximately 200 units per mg. Such preparations also contain small amounts of oxytocic activity. As mentioned previously, however, the accuracy of oxytocic assays in the presence of such large amounts of pressor activity is open to question. Moreover, the

TABLE 2

PURIFICATION PROCEDURE OF STEHLE AND FRASER[4]

Fraction	Weight	Procedure	Potency, Units per mg.		Total, Activity	
			Pressor	Oxytocic	Pressor	Oxytocic
	gm.				*Per cent*	*Per cent*
A	1000	Acetone desiccated posterior lobes	1	1	100	100
		Extracted with hot 0.5 per cent HAc; concentrated; treated with absolute ethanol. ↓				
B	218	Alcohol precipitate	4	4	87	87
		Dissolved in 0.5 per cent H_2SO_4; $Ba(OH)_2$, $Fe_2(SO_4)_3$ and dialysed iron added; filtrate concentrated to dryness; dissolved in 96 per cent methanol; fractionated with EtAc, repeated twice				
C	7.3	Precipitate Fractionated from ethanol or methanol with EtAc.	100	30	73	22
D (Pressor)	2.0	Precipitate ↓ Methanol-EtAc mother-liquor fractionated with methanol, ethanol and EtAc.	200	10	40	2
E (Oxytocic)	0.55	Precipitate	4	250	0.2	14

TABLE 3

PURIFICATION PROCEDURE OF IRVING, DYER, AND DU VIGNEAUD[5]

Fraction	Weight	Procedure	Potency, Units per mg.		Total Activity	
			Pressor	Oxytocic	Pressor	Oxytocic
	gm.				Per cent	Per cent
A	6000	Frozen posterior lobes Ground with sand and water; juice separated under pressure; juice acidified, boiled 10 min., centrifuged; supernatant concentrated; saturated with NaCl.	0.2	0.2	100	100
B	200	Precipitate, dried Extracted with 98 per cent HAc; ether and petroleum ether added to extract.	4	4	80	80
C	78	Precipitate Dissolved in 98 per cent HAc; ether added; repeated once.	9	9	72	75
D	58	Precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether to give several precipitates.	9	5	52	27
E	5.2	Most potent precipitate Dissolved in 98 per cent HAc; fractionally precipitated with acetone and ether as above.	55	30	29	16
F	1.5	Most potent precipitate Dissolved in glacial HAc; precipitated with ether; repeated 3 times.	85	30	13	4.5
G	1.1	Precipitate Dissolved in water; extracted with butanol.	110		12	
H	0.6	Aqueous solution Electrophoresis, and isolation of cathode material.	125	20	8.1	1.3
I (Pressor)	0.2	Precipitate	200	26	4.0	0.5
		HAc-ether mother-liquor treated with water and petroleum ether.				
J (Oxytocic)		Gummy precipitate	22	146	5	34

possibility cannot be ignored that the pressor principle itself may possess slight inherent oxytocic properties. A similar possibility must also be admitted regarding the presence of pressor activity in oxytocic preparations. The most potent oxytocic preparation obtained has an activity of 250 units per mg. By procedures not fully described, Kamm and his coworkers[3] state that they were able to prepare an oxytocic fraction having an activity of 350 units per mg. In a later paper[29] it was indicated that potencies as high as 500 oxytocic units per mg. had been attained. The pressor and oxytocic preparations described recently by Potts and Gallagher[28] contain 450 and 700 units per mg., respectively.

The purified pressor and oxytocic preparations are white or slightly cream colored, amorphous powders. Hygroscopicity varies but is usually insufficient to cause trouble in weighing or handling under ordinary conditions. The activity of these dry preparations is quite stable for periods of at least several months.

CHEMICAL AND PHYSICAL PROPERTIES OF THE SEPARATE PRESSOR AND OXYTOCIC PREPARATIONS

Analysis

The first significant analytical study of posterior lobe hormone preparations was undertaken by du Vigneaud and his associates in 1933[29] when it was shown that these preparations contain relatively large amounts of cystine sulfur and tyrosine. These data together with analytical figures obtained subsequently in other laboratories are given in TABLE 4. Data are included for several purified pressor and oxytocic preparations and for a few preparations of low potency. The differences between the analytical values for a given constituent may appear to be great but, inasmuch as the materials analysed were prepared by different procedures and the determinations made by different methods, such variations are not surprising. The figures indicate that highly purified pressor preparations contain large amounts of cystine, tyrosine and arginine, while highly purified oxytocic preparations contain closely similar amounts of cystine and tyrosine but considerably less arginine. Unfortunately, the preparations analysed are not pure substances. Consequently, the analysis of a few preparations provides no sound basis for predicting the composition of the principles themselves.

Very limited but more indicative information concerning the probable composition of the principles has been gained by the systematic analysis

[29] du Vigneaud, V., Sealock, R. R., Sifferd, R. H., Kamm, O., & Grote, I. W. Proc. Soc. Biol. Chem., Jour. Biol. Chem. 100: p. xciv. 1933.

TABLE 4

ANALYSIS OF PRESSOR AND OXYTOCIC PREPARATIONS

Potency of Preparation		N	S	NH₂-N	Cys-tine	Tyro-sine	Argi-nine	Investigators	Remarks
Pressor Units per mg.	Oxytocic Units per mg.	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent		
200	?		3.1	–		10.5		du Vigneaud and co-workers[29]	Values corrected for ash and moisture.
?	500		3.1	–	9.0	14.3			
200	6	15.6	3.4			11.5	3.8	Sealock[30]	Values corrected for ash and moisture.
2	500	15.2	3.3		9.6	15.8	-		
200	8-10	13.9	3.0	2.0	7.7	9.5	8.9	Stehle and Fraser[1]	Moisture and ash corrections not indicated. Average values.
4	250	13.8	3.6	2.5	8.9	10.7	6.1		
4	4	13.7	1.6	1.2		2.6	8.6		
200	25	14.3		–	11.2	9.9	–	Irving, Dyer, and du Vigneaud[3]	Not corrected for ash or moisture.
9	9	14.6		–	10.8	2.2	–		
?	240	14.0	3.2	1.4	–			Freudenberg, Weiss, and Biller[31]	Moisture and ash corrections not indicated.
450	<40	–	–	–	19.0	11.9	12.3	Potts and Gallagher[28]	Moisture and ash corrections not indicated.
<20	700	–	5.6	–	18.3	14.2	<0.8		

[30] Sealock, R. R., A thesis submitted to the George Washington University, 1935.
[31] Freudenberg, K., Weiss, E. & Biller, H. Z. physiol. Chem. 233:172. 1935.

of preparations of different potencies. Data obtained in this manner for both pressor and oxytocic preparations show that the sulfur content of these preparations increases progressively as the pressor or oxytocic potency increases, indicating that both principles undoubtedly contain sulfur.[29] Likewise analytical figures for tyrosine and cystine strongly indicate the presence of these two amino acids in the pressor principle[5] as shown in FIGURE 1. Similar analyses for arginine in both principles

Pressor units per mg.
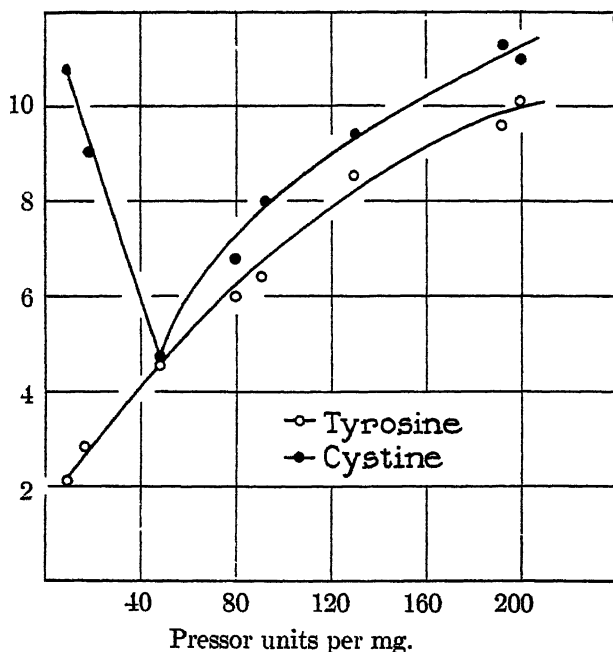
FIGURE 1.

and for cystine and tyrosine in the oxytocic principle have not been carried out.

Stehle and Trister[32] have recently examined purified pressor and oxytocic preparations for a large number of amino acids. These data are tabulated in TABLE 5. In this work, rather large amounts of each of the purified preparations were hydrolyzed in 20 per cent HCl, and attempts were made to isolate certain amino acids or their derivatives from the hydrolysates. Where the results were positive, the yields were sufficiently large to permit purification and characterization of the pro-

³³ Stehle, R. L., & Trister, S. M. Jour. Pharmacol. **65**: 343. 1939.

TABLE 5

IDENTIFICATION OF AMINO ACIDS IN HYDROLYSATES OF PRESSOR AND OXYTOCIC PREPARATIONS[32]

Amino acid	Characterized by	Pressor preparation	Oxytocic preparation
Tyrosine	Picrolonate	+	±*
Cystine	Amino acid, hydantoin	+	±*
Arginine	Flavianate	±	+
Proline	Reineckate	+	+
Isoleucine	Hydantoin	+	—
Leucine	Hydantoin	—	+
Histidine	Colorimetric test	—	—*
Glutamic acid	Hydrochloride	—	—
Phenylalanine	Picrolonate	—	—
Hydroxyproline	Reineckate	—	—
Glycine	Orthophthalic aldehyde	—	—
Tryptophane	Colorimetric test	trace	trace
Cysteine	Colorimetric test	—	—

* Freudenberg and coworkers found tyrosine, cystine and histidine in the hydrolysate of an oxytocic preparation. 240 units per mg.

duct. Qualitative tests were also applied in some cases. These results show that tyrosine, cystine, arginine, and proline can be isolated from hydrolysates of both pressor and oxytocic preparations. Isoleucine seems to be present only in the pressor fraction while leucine appears to be present only in the oxytocic fraction. Stehle and Trister failed to find significant amounts of histidine, glutamic acid, phenylalanine, hydroxyproline, glycine, or tryptophane in either preparation. No tests have been made to determine the presence or absence of alanine, aspartic acid, hydroxyglutamic acid, lysine, methionine, serine, threonine, or valine. With respect to the positive results in TABLE 5, it should be re-emphasized that since the purity of the preparations examined has not been established, inferences regarding composition of the principles themselves must be made with caution.

Freudenberg and Biller[33] have reported the isolation of choline as the reineckate from a sulfuric acid hydrolysate of an oxytocic preparation containing 250 oxytocic units per mg. and estimate from the yield that more than 1 per cent of choline could be present in the oxytocic principle. Prompted by this observation, and the hypothesis that the oxytocic principle might be a choline ester of an amino acid or peptide, Freudenberg and Keller[34] and later Gulland, Partridge and Randall[35] synthesized many choline esters of this type but, so far, none of the synthetic products exhibits significant oxytocic activity. Apparently the possibility was not

[33] Freudenberg, K., & Biller, H.   Naturwissensch. 24: 523. 1936.
[34] Freudenberg, K., & Keller, R.   Ber. 71B: 320. 1938.
[35] Gulland, J. M., Partridge, M. W., & Randall, S. S.   Jour. Chem. Soc. p. 419. 1940.

considered that the choline might have been present as an impurity in the original oxytocic preparation.

In purified pressor preparations choline is absent.[36] We have found that hydrolysates of highly purified pressor fractions exhibit a powerful depressor action upon mammalian blood pressure, a fact which is suggestive of choline. This action is not appreciably increased by acetylation nor is it blocked by atropinization under conditions in which controls with added choline respond in the expected manner. Furthermore, injections of large amounts of an unhydrolyzed pressor fraction into an animal which has been made refractory to the pressor principle by rapidly repeated injections, produce a depressor response that is qualitatively and quantitatively identical with that obtained by using an equivalent amount of hydrolysate of the same pressor fraction. In other words, the depressor action of the unhydrolyzed material becomes evident when the masking effect of the predominant pressor action is eliminated either by hydrolysis or by test upon a pressor-refractory animal. In our opinion these observations show conclusively that the substance responsible for the depressor action of hydrolyzed pressor preparations is not choline and further, that this substance is not an integral part of the pressor molecule. Tests upon fractions obtained at various stages in the purification of the pressor principle show that this depressor impurity is present in relatively large amounts in crude gland extracts and that it is partially eliminated in the purification process. From its solubility and pharmacological behavior it is likely that the substance may be histamine, which has been isolated from posterior lobe extracts.[37] Only 0.01 mg. of histamine per 100 mg. of potent pressor preparation could account for the depressor effects observed.

A similar study of potent oxytocic preparations would be of definite value in clarifying the present status of the oxytocic principle with respect to choline.

Molecular Weight

With the possible exception of choline, significant amounts of substances other than amino acids have not been found in hydrolysates of either principle. Furthermore, elementary analysis indicates the presence of approximately 15 per cent nitrogen and 3 per cent sulfur. These facts, together with the information concerning solubility behavior gained from purification studies, have naturally led to the supposition that the principles themselves are either proteins or polypeptides. Con-

[36] du Vigneaud, V., & Irving, G. W., Jr. Unpublished data.
[37] Abel, J. J., & Kubota, S. Jour. Pharmacol. **13**: 243. 1919.

siderable evidence is available to substantiate this view. Positive biuret and ninhydrin color reactions are obtained with purified preparations of both principles. Acid hydrolysis rapidly destroys pressor, oxytocic and antidiuretic activity with the simultaneous liberation of amino acids. Hydrolysis of potent pressor and oxytocic preparations results in a four- to fivefold increase in amino nitrogen. Certain proteolytic enzymes rapidly destroy all three activities.

It may be concluded, therefore, that the molecules responsible for the activities of pressor and oxytocic preparations contain amino acids joined in peptide linkage, and that the presence of the intact peptide structure is essential for the pharmacological activity of these molecules. Since it is obvious that either a peptide or a protein could satisfy these requirements additional evidence must be examined to determine the approximate size of the active molecules.

As early as 1899 Schäfer and Vincent[18] discovered that pressor activity is not retained by dialyzing membranes. From this observation, which has been confirmed and extended to include the oxytocic and antidiuretic activities, it is evident that the size of the molecules responsible for these activities is of a much lower order of magnitude than even the simplest proteins. Approximations of molecular size have been obtained by comparing the rate of dialysis of the principles with the rates for substances of known molecular weight. Smith and McClosky[19] found that all three activities passed rapidly through a collodion membrane which was completely impermeable to trypan blue (mol. wt. 932). The diffusion rates for the two principles were approximately equal and agreed closely with the rate for methylene blue (mol. wt. 374). Kamm[40] found that the pressor and oxytocic principles diffused through collodion at about half the rate of adrenalin (mol. wt. 183) and estimated the molecular weights of each of the two principles to be in the neighborhood of 600. On the basis of ultracentrifuge studies Rosenfeld[41] agrees that Kamm's estimate is probably of the correct order of magnitude. Since the pressor and oxytocic activities contained in commercial preparations of the two principles (pitressin and pitocin), showed but little tendency to sediment after nearly 6 hours of centrifuging at 61.000 r.p.m. Molecular weight estimates have also been made from analytical data. Stehle and Fraser find that the simplest molecular weights which can be calculated from the sulfur contents of the purified principles, assuming that the sulfur is present as cystine sulfur, are 1778 for the oxytocic and 2100 for the pressor principle. Assuming 1 amino group per molecule a

18 Schäfer, E. A., & Vincent, S. Jour. Physiol. 25: 87. 1899.
19 Smith, M. I., & McClosky, W. T. Jour. Pharmacol. 24: 371. 1924.
40 Kamm, O. Science 67: 199 1928.

molecular weight of 700 can be calculated from Stehle and Fraser's figures which indicate the presence of approximately 2.0 per cent amino nitrogen in the unhydrolyzed preparations. Potts and Gallagher[28] find that the cystine and tyrosine values for their 700 unit oxytocic fraction are in good agreement with a minimum molecular weight of 1300 for the oxytocic principle, assuming the presence of one molecule of each amino acid. On the same basis, a closely similar minimum molecular weight can be calculated for their 450 unit pressor fraction.

The evidence presented so far indicates (1) that the two active principles are polypeptides; (2) that they have molecular weights between 600 and 2000; and (3) that they probably contain cystine, tyrosine, and arginine and possibly proline and leucine or isoleucine. Aside from the very significant fact that the highly purified oxytocic preparation of Potts and Gallagher[2b] seems to contain considerably less arginine than their purest pressor fraction none of the evidence presented so far reveals any striking chemical or physical differences between the two principles nor does it indicate what parts of the molecules are necessary for their pharmacological activity. A number of approaches have been used in an attempt to throw additional light upon these questions.

Action of Enzymes

The action of proteolytic enzymes has been investigated almost entirely from the standpoint of their effects upon the activity of purified preparations. Trypsin preparations rapidly destroy pressor, oxytocic and antidiuretic activity whereas pepsin has no effect upon any of them.[41–47] Results with papain are in disagreement but it is probable that this enzyme destroys oxytocic activity.[44,45,48] Extracts of kidney, liver, muscle, blood, yeast, and intestinal mucosa destroy pressor and oxytocic activity.[45,49] Gulland finds that aminopolypeptidase, dipeptidase, and proteinase preparations from yeast destroy oxytocic activity, but believes that the action is due to a separate enzyme present in each of these preparations and also present in trypsin preparations. Larson[49] believes that aminopolypeptidase alone is responsible for the destruction of pressor activity, dipeptidase being without effect.

Besides establishing the inactivating effect of enzymatic hydrolysis of peptide bonds, these observations provide little information concerning

1 Dale, H. H.   Biochem. Jour. 4: 427.   1909.
2 Dudley, H. W.   Jour. Pharmacol. 14: 295.   1919.
3 Thorpe, W. V.   Biochem. Jour. 20: 374.   1926.
4 Freudenberg, K., Weiss, E., & Eyer, H.   Naturwissensch. 20: 658.   1932.
5 Gulland, J. M., & Macrae, T. F.   Nature 131: 470.   1933.
6 Gulland, J. M., & Macrae, T. F.   Biochem. Jour. 27: 1237.   1933.
7 Gulland, J. M., & Macrae, T. F.   Biochem. Jour. 27: 1383.   1933.
8 Dale, H. H., & Dudley, H. W.   Jour. Pharmacol. 18: 27.   1921.
9 Larson, E.   Jour. Pharmacol. 62: 346.   1938.

the nature of the active molecules, but the difference between the action of trypsin and pepsin suggests that more might be learned by this approach. A careful study of the pituitary principles with a number of specific proteolytic enzymes might reveal certain structural possibilities or differences.

Other enzymatic studies indicate that prolinase and arginase exert no effect upon the activity of the oxytocic principle,[47] but tyrosinase causes complete inactivation.[48] This obervation points to the possible essentiality of the tyrosine phenolic grouping to the activity of the oxytocic principle.

Action of Various Chemical Agents

Investigation of the effects of various chemical and physical treatments upon the activity of the pressor and oxytocic principles has provided material for speculation but very little concrete information as to the structure of the hormones themselves, as shown in TABLE 6.

It is well known that the principles are quite stable in dilute acid pH 3.0 .[49] At this pH the principles may be boiled for several minutes without affecting their activity. It is equally well recognized that the principles are quite unstable in alkaline solution. Exposure to $1 \times$ alkali for a few hours at room temperature is sufficient to destroy completely both pressor and oxytocic activity[42], and more vigorous treatment with alkali is accompanied by the liberation of hydrogen sulfide.[43]

The action of many chemical agents upon the activity of purified oxytocic preparations has been extensively studied in the laboratories of Kamm.[52, 53] Guha[54–57] and Gulland[58–60], but the experimental conditions used have varied so widely that it is hazardous to base any conclusions regarding chemical structure upon an analysis of the assembled data. Under these circumstances it is preferable to present the authors' interpretations as to the significance of their results.

Kamm and coworkers were of the opinion that such reagents as Na_2SO_3. $NaHSO_3$. $Na_2S_2O_3$. $Na_2S_2O_4$ and SO_2 not only destroy pressor and oxytocic activity but that they convert the pressor principle into a

[49] Gaddum, J. H. Biochem. Jour. 24: 939. 1930.
[50] Sullivan, M. X., & Smith, M. I. Public Health Reports. U. S. P. H. S. 43: 1934. 1928.
[51] Kamm, O., Grote. I. W., & Rowe, L. W. Proc. Soc. Biol. Chem., Jour. Biol. Chem. 92: p. lxiv. 1931.
[52] Kamm, O., & Grote I. W. Canadian Patent 367,624, July 27, 1937, Chem. Abs. 31: 6923. 1937.
[53] Guha. B. C., & Chakravorty, P. N. Indian Jour. Med. Res. 21: 429. 1933.
[54] Das, N., & Guha, B. C. Indian Jour. Med. Res. 21: 765. 1934.
[55] Das, N., & Guha, B. C. Indian Jour. Med. Res. 22: 157. 1934.
[56] Das, N., & Guha, B. C. Indian Jour. Med. Res. 22: 317. 1935.
[57] Gulland, J. M. Biochem. Jour. 27: 1218. 1933.
[58] Gulland, J. M., & Randall, S. S. Biochem. Jour. 29: 378. 1935.
[59] Gulland, J. M., & Randall, S. S. Biochem. Jour. 29: 391. 1935.

"derived" hormone having a very low sulfur content and possessing antidiuretic activity. The chemical changes involved were not discussed nor have the experimental findings been confirmed. Gilman and Goodman[12] mention the fact that treatment with sodium sulfite does not cause loss of antidiuretic activity.

Guha and his collaborators tested the effects of H_2O_2, HNO_2, HNO_3, Br_2, SO_2, and benzoyl and acetyl chlorides upon the activity of the oxytocic principle but refrained from discussing the structural implications of their results. Inactivation was obtained in all cases but the absence of adequate controls makes their estimates of the degree of inactivation questionable.

TABLE 6

ACTION OF VARIOUS AGENTS UPON PURIFIED PRESSOR AND OXYTOCIC PREPARATIONS

Agent	Pressor	Oxytocic
Acid hydrolysis	Inactivated	Inactivated
Alkali, cold	"	"
Na_2SO	"	"
$NaHSO_3$	"	?
$Na_2S_2O_5$	"	?
$Na_2S_2O_4$	"	?
HNO_2	?	Inactivated
NaCN	?	"
H_2O_2	?	"
I_2, Cl_2, Br_2	?	"
Ultraviolet light	?	"
SO_2	Inactivated	Partial to complete inactivation
HCN	?	Partially inactivated
Benzoyl chloride	?	" "
Acetyl chloride	?	" "
Electrolytic reduction	?	Partially inactivated (irreversible)
Catalytic reduction	?	Partially inactivated (irreversible?)
H_2S	?	Partially inactivated (reversible)
Semicarbazide	?	Unaffected
Hydroxylamine	?	"
Cysteine reduction	Unaffected	"
Cysteine reduction followed by benzylation	Inactivated	Inactivated

Gulland and coworkers observed that HNO_2 completely inactivates the oxytocic hormone and believed that three separate reactions are involved in the inactivation process. In the first step nitrous acid is presumed to convert the principle rapidly into a derivative possessing 35 per cent of the original potency. This derivative is then converted into a second intermediate having a potency of 20 per cent and finally

into a substance having no oxytocic activity. No chemical changes were
suggested to explain this behavior.

Gulland and Randall found that inactivation by reduction could be
reversed in some cases by oxidation but that the degree of reactivation
depended largely upon the reducing conditions used and the extent of the
reduction. Thus, an oxytocic preparation which had been 50 per cent
inactivated through the action of H_2S for 24 hours could be completely
reactivated by treatment with oxidized methylene blue at pH 4.2. On
the other hand, when the same degree of reductive inactivation was
accomplished catalytically or electrolytically, treatment with oxidized
methylene blue caused only partial reactivation. By using a series of
oxidation-reduction dyes the oxytocic principle was found to contain a
redox system having a potential of $E_0' = +0.025$ volts at pH 6.0. When
the system is completely oxidized the hormone is considered to be fully
active; when fully reduced the activity is 50 per cent. Attempts were
made to elucidate the structure of this redox system by studying the
effects of the following reagents upon oxytocic activity: Na_2SO_3 at pH
8.4, SO_2 at pH 5.5, $NaCN$ at pH 7.8, HCN at pH 3.5, H_2O_2 at pH 3.5
and 8.2, I_2 at pH 7.5, Cl_2 in acid solution, semicarbazide, and hydroxyl-
amine. Although these workers were unable to correlate their data
with any definite structure they suggested that none of the results was
opposed to the view that a disulfide linkage could be present in the hor-
mone molecule if the corresponding reactions of cystine were used as an
analogy. Thus, cystine is reduced by Na_2SO_3 and by $NaCN$ and these
compounds inactivate the oxytocic principle. Similarly, cystine is
oxidized to cysteic acid by iodine and chlorine and the halogens also in-
activate the hormone. Gulland and Randall are forced to admit, how-
ever, that SO_2 and HCN, which exert no effect upon cystine, both cause
appreciable inactivation. Furthermore, H_2O_2 which rapidly oxidizes
cystine had a most peculiar action upon the oxytocic principle. In
either acid or alkaline solution, H_2O_2 first caused 50 per cent inactiva-
tion, then a reactivation to 90 per cent, and finally complete destruction.
In view of these facts Gulland and Randall felt that they were not justi-
fied in making definite the suggestion that a disulfide linkage is present
in the oxytocic molecule.

Gilman and Goodman[12] claim that antidiuretic activity is resistant
to the reducing agents found by Gulland and coworkers to inactivate
the oxytocic hormone.

The chemical information available in 1935 suggested to Sealock and
du Vigneaud[61] certain similarities between the posterior lobe principles

[61] Sealock, R. R., & du Vigneaud, V. Jour. Pharmacol. 54: 433. 1935.

and insulin. With the latter hormone it had been demonstrated[62] that reduction of disulfide linkages in the molecule to the sulfhydryl form by means of cysteine or reduced glutathione resulted in irreversible destruction of hormonal activity. This technique seemed to provide a method for demonstrating whether or not the sulfur of posterior lobe preparations was actually present as a disulfide in the active principles and if so, whether or not reduction of this linkage caused inactivation. Solutions of highly purified pressor and oxytocic preparations, buffered at pH 8.0, were treated with an excess of cysteine at room temperature in an atmosphere of nitrogen for periods of 2 to 48 hours. With both preparations the reduced products and the products obtained by reoxidation were found to be as active pharmacologically as the original preparations. In view of these results proof was needed that reduction by cysteine had been accomplished. It was reasoned that if the principles originally contained disulfide linkages, then the sulfhydryl groups formed on reduction should be susceptible to benzylation or methylation. When the original preparations were treated with benzyl chloride or methyl iodide no change in activity resulted. But when the reduced solutions were treated similarly, until the test for sulfhydryl groups was negative, all pressor and oxytocic activity was destroyed. In the latter experiments assays made at intervals during the benzylation showed that the activity decreased gradually over a 10 hour period, indicating that the inactivation was undoubtedly the result of the benzylation reaction. Suitable controls were run to show that the pH of the medium caused no injury to the active principles and that the reagents used did not affect the assay animals.

The authors felt that the combination of cysteine reduction and benzylation, together with the effects of these treatments on pharmacological activity, offered almost conclusive proof that the active principles contain a disulfide linkage and that a sulfhydryl group or a potential sulfhydryl group is essential to the activity of these hormones.

The validity of these conclusions was questioned by Gulland and Randall[63] who maintained that cysteine had not been adequately established as a specific reducing agent for the disulfide linkage and consequently, results obtained through its use did not necessarily prove the presence of such a linkage. It was claimed further that the behavior of the hormones to reduction and benzylation could be explained on the basis of a number of redox systems, the reduced forms of which might

[62] du Vigneaud, V., Fitch, A., Pekarek, E., & Lockwood, W. W. Jour. Biol. Chem. **94**: 233. 1931.

[63] Gulland, J. M. & Randall, S. S. Jour. Soc. Chem. Ind. **55**: 442. 1936.

be susceptible to benzylation with attendant loss of activity. Such reduced groups as -OH and -NH- were suggested as possibilities.

In reply to the criticism of Gulland and Randall, du Vigneaud[1] emphasized the fact that the conclusions drawn from the reduction experiments were not based upon the specificity of the reduction alone but upon the combination of reduction with cysteine and then inactivation by benzylation after reduction. In other words, it would be necessary to visualize some grouping in the hormones which would be capable of being reduced by cysteine at a neutral pH at room temperature and which, only after reduction, would be benzylated under the conditions used. No grouping other than the disulfide could be found to fit these requirements. Consequently, du Vigneaud felt that there is reason to believe that a disulfide does exist in the pressor and oxytocic hormones; that reduction of this group is obtained by treatment with cysteine; and that the sulfhydryl forms of these hormones are active.

Partial support for the conclusions of Sealock and du Vigneaud was provided by Freudenberg, Weiss and Biller[31] who found that the oxytocic hormone remained fully active after treatment with sodium amalgam in alkaline solution even though sulfhydryl groups were liberated. These workers also provided corroboration for the destruction of oxytocic activity by H_2O_2, alkali, neutral or alkaline sulfite, neutral or alkaline iodine, catalytic reduction and ultraviolet light. However, acid iodine was found to be without effect. Partial reactivation of a catalytically reduced oxytocic preparation was secured by peroxide oxidation. In those cases in which the treatment caused inactivation of the oxytocic hormone, Freudenberg claimed that the reaction proceeded less rapidly than did the inactivation of insulin under similar conditions.

Certain aspects of the foregoing results require further examination. If we accept the view that a disulfide linkage is an integral and essential part of both hormone molecules, the inactivating effects of oxidizing agents such as H_2O_2 and the halogens can be explained entirely on the basis of an oxidative destruction of the disulfide linkage. Oxidation of any other susceptible groups in the molecule can, of course, also occur. If we accept the view that reduction of the disulfide linkage to the sulfhydryl form causes no change in activity, the inactivating effects of a number of the reducing agents previously discussed must be explained on some other basis. Reagents like cyanide or sulfite inactivate the hormone and are also capable of reducing disulfide linkages. Because it is believed that such a reduction alone does not cause inactivation, it must be concluded that the inactivation caused by these reagents is due

51 du Vigneaud, V.   Cold Spring Harbor Symposia on Quant. Biol. VI: 275.   1938.

to their simultaneous reduction of, or reaction with, additional groups in the hormone molecules. This explanation is all the more probable since it is obvious that the disulfide linkage cannot be the only group necessary for the activity of the hormone. SO_2, HCN and HNO_2, which exert no effect upon the disulfide linkage, can be considered to cause their inactivating effects by action upon these unknown groups. Though our knowledge concerning the amino acids probably present in the active principles and the behavior of the hormones to certain chemical treatments provide clues for future investigation, it is as yet much too inadequate to enable us to decide which ones of the several possible groups are involved in these inactivation reactions.

Electrophoresis Experiments

Investigation of the electrophoretic behavior of the pressor and oxytocic principles has yielded some very useful information concerning the electrochemical nature of these hormones. It will be recalled that much of the early chemical work indicated that both principles were predominantly basic in character. In fact Kamm and his associates[3] were led to conclude from their purification studies that both active principles "appear to be basic in character," and accordingly named their isolated products, alpha and beta hypophamine, to signify that they might be amines derived from the hypophysis. The electrodialysis studies of Freeman, Gulland and Randall,[65] and later those of Das, Ghosh and Guha[66] upon purified oxytocic preparations did little to change this view. These investigators found that although migration of the hormone toward the cathode was obtained readily under certain conditions, migration toward the anode did not occur. It was concluded, therefore, that the oxytocic principle was either a base or was adsorbed on basic material.

An observation made in the Cornell laboratory in the course of studies on the purification of the posterior lobe principles by electrophoresis strongly indicated that the hormones are not bases but amphoteric molecules.[5] It was observed during electrophoresis that neither principle was able to enter a compartment where the pH was maintained at 12. Accordingly, in cooperation with Dr. Mildred Cohn,[11] a detailed study of the migration of the pressor principle over a wide pH range was carried out in an apparatus designed for this purpose and in the apparatus of Tiselius. The direction and extent of migration of pressor activity after suitable periods of electrophoresis was determined by assay and the net mobility was calculated. The results plotted in FIGURE 2 show

[65] Freeman, M., Gulland, J. M. & Randall, S. S. Biochem. Jour. **29:** 2211. 1935.
[66] Das, N., Ghosh, B. N. & Guha, B. C. Zeit. physiol. Chem. **238:** 131. 1936.
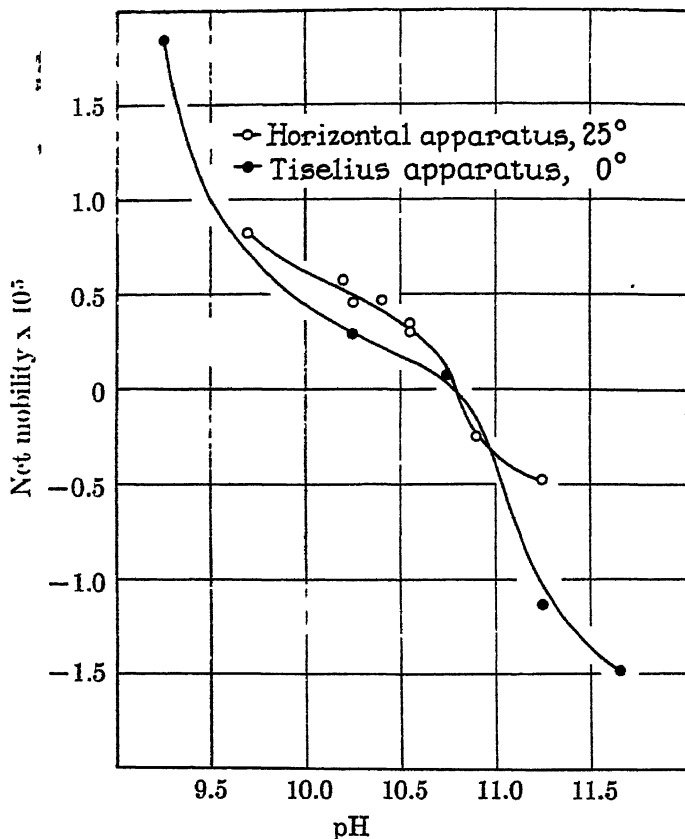
FIGURE 2.

that the pressor principle is definitely amphoteric with an isoelectric point at about pH 10.8 in buffers of 0.02 ionic strength. A preliminary investigation of highly purified oxytocic preparations indicates that this principle is also amphoteric and that it has an isoelectric point in the region of pH 8.5.

It should be mentioned here that in our procedure for the purification of the pressor hormone (TABLE 3), advantage was taken of its high isoelectric point to prevent destruction of activity by cathodic reduction during electrophoresis. The negative electrode was placed in a cell having a pH of 12. Since in migrating toward the cathode the amphoteric principles were unable to enter a solution having a pH above their isoelectric points. electrode reactions were eliminated.

THE UNITARY HORMONE CONCEPT

(ISOLATION OF AN ACTIVE PROTEIN FROM THE POSTERIOR LOBE)

As stated earlier, Abel and his group believed that the pharmacological activities of the posterior lobe were due to a single, large "mother-molecule." This view was maintained even after the experiments of Dudley and Kamm had shown that separate pressor and oxytocic preparations could be obtained from posterior lobe extracts by suitable fractionation procedures. Abel insisted that in such separations the treatments used were sufficiently drastic to cleave the "mother-molecule" producing several small active fractions. The definition of the unitary theory on this basis placed its proponents in a very difficult position because the proof of such an hypothesis rested upon the successful isolation of the supposed "mother-molecule," the demonstration that it possessed all of the pharmacological properties of the posterior lobe, and that it could be split by the suspected treatments to give separate pressor and oxytocic principles. Professor Abel was unable to isolate the hypothetical "mother-molecule" and based his convictions chiefly on his ability to obtain purified preparations in which the ratio between the pressor, oxytocic and melanosome-dispersing activities was the same as that found in untreated gland extracts. But, when it became evident that melanosome-dispersing activity was not a property of the posterior lobe, the basis for Abel's argument became less secure. Valid evidence was also advanced to show that many of Abel's preparations were not the partially purified "mother-molecule" he suspected but mixtures of the separate principles since the activity ratio was occasionally not identical with that of untreated extracts. In some instances, however, the ratio between the various activities accidentally coincided with that found in the gland. Nevertheless, it was realized that Abel's contentions regarding the possibility of a "mother-molecule" could by no means be disregarded.

In 1931 MacArthur[67] sketchily outlined a process for obtaining an active preparation from the posterior lobe which seemed to fit some of the requirements of the hypothetical "mother-molecule," but too few details were given to permit a definite conclusion. The work was never confirmed or extended. The preparation was obtained by acetone and ether precipitation from a 70 per cent methanol (containing 5 per cent acetic acid) extract of dessicated fresh glands. MacArthur's product was a sparingly water soluble substance, possessing both pressor and oxytocic activity and having an isoelectric point at about pH 5.0. Ac-

tivity was destroyed by acid hydrolysis and by trypsin. It was unstable in weak alkali and contained labile sulfur.

At this stage in the development of the posterior lobe problem new methods of approach were desired to throw additional light upon the unitary and multiple hormone theories. Electrophoresis seemed to provide such a method.[8] The apparatus used consisted essentially of a series of beakers filled with water and joined by means of inverted U-shaped siphons. Electrodes were placed in the end cells and the pituitary material was dissolved in one of the cells. After a suitable period of electrophoresis the contents of each cell were assayed for pressor and oxytocic activity. When slightly acid solutions of partially purified posterior lobe preparations containing both principles were subjected to electrophoresis in a 19 cell train, it was found that both principles migrated toward the cathode and that the rate of migration of pressor activity was approximately six times that of oxytocic activity. Since the two activities migrated at different rates it was concluded that they were physiological manifestations of different chemical entities in the preparation electrolyzed, a fact which was in agreement with the preponderance of evidence then available.

By means of the same technique it was possible to study also the electrophoretic behavior of the activities contained in the mechanically expressed juice of fresh, untreated glands.[10] Posterior lobes were removed from steers within a few minutes after the animals were killed and were immediately frozen in a tube immersed in dry ice. The glands were thawed, ground with sand and a little water, and the juice was separated by application of high pressure. The juice (pH 6.0, 560 pressor and 420 oxytocic units was placed in the center cell of a 5-cell setup and electrophoresis was carried out for 17 hours. The time elapsing from the removal of the glands to the end of the electrophoresis was 23 hours, during which time the temperature of the glands and juice was never above 10° C. The results which are given in TABLE 7 show that pressor activity traveled at a faster rate than oxytocic activity, demonstrating that these activities in the simple press juice are attributable to different chemical entities.

We believe that this conclusion was justified since the material studied was subjected to no chemical treatments and since the possibility of preferential destruction of one activity was eliminated by quantitative recovery of both activities. It was realized, however, that these results could not be taken to indicate that the same situation necessarily existed in the gland itself. The elimination of such a possibility was beyond the scope of this approach.

TABLE 7

DISTRIBUTION OF ACTIVITY AFTER ELECTROPHORESIS OF POSTERIOR LOBE PRESS JUICE

Cell number	Pressor units	Oxytocic units	pH at end of experiment
1 (cathode)	27	3	10.2
2	432	240	6.2
3 (starting)	96	170	4.0
4	3	9	3.0
5 (anode)	0	0	2.3
	558	422	

It is only quite recently that evidence favorable to the unitary hypothesis has been advanced. In 1940 Rosenfeld[16] compared the behavior in the ultracentrifuge of purified pressor and oxytocic preparations with that of untreated press juice from posterior lobes. The purified pressor and oxytocic preparations showed very little tendency to sediment even after almost 6 hours at 61,000 r.p.m. Rosenfeld concluded that the two activities present in such preparations could therefore be attributed to small molecules which might well be of the order of 600 molecular weight. the value estimated by Kamm.[40] On the other hand, both activities sedimented rapidly and at approximately the same rates when press juice was centrifuged. It was concluded that the two activities present in the press juice resided either in a single large molecule (the unitary principle of Abel), or in two separate large molecules having about the same sedimentation rates. The molecular size of the native posterior lobe hormone was provisionally placed at 20,000 to 30,000. To indicate the effects of acid treatment at elevated temperature on the lability of the hormone molecule, a sample of the press juice was mixed with an equal volume of 0.5 per cent acetic acid. brought quickly to boiling. cooled and filtered. Ultracentrifugation of the filtrate showed the presence of both slowly and rapidly sedimenting active particles. Rosenfeld concluded from this result that a portion of the native hormone had been split by the treatment into small, active fragments.

In the foregoing experiment, as in many experiments where claims for the demonstration of a large active molecule have been made, the possibility of adsorption of smaller, extremely active substances upon a biologically inert substance cannot be ignored. In Rosenfeld's work the possibility that adsorption could account for his results with press juice has not been entirely eliminated. In this respect experiments in which known amounts of activity in the form of low molecular weight preparations are added to the press juice before centrifuging would be

TABLE 8

PURIFICATION PROCEDURE OF VAN DYKE AND HIS COLLABORATORS[68]

Fraction	Weight	Procedure	Potency, units per mg.		Total activity	
			Pressor	Oxytocic	Pressor	Oxytocic
					Per cent	Per cent
A	1 kg.	Fresh posterior lobes Ground and suspended in 0.01N H₂SO₄, pH 4.25	0.2	0.2	100	100
B	—	Supernatant from centrifuging pH adjusted to 3.9; 80 gm. NaCl added per liter.	—	—	75–80	75–80
C	—	Precipitate Dialized Cl⁻ free; 10 gm. NaCl added per liter; pH 3.5	—	—	50	50
D	—	Supernatant from centrifuging Equal vol. M 1 acetate buffer, pH 3.9 added; 20 gm. NaCl added per liter	—	—	—	—
E	—	Supernatant from centrifuging and washing. 40 gm. NaCl added per liter.	—	—	—	—
F	—	Precipitate Dissolved in minimum volume of water; equal vol. M 1 acetate buffer added; 6.5 gm. NaCl added per 100 cc.	—	—	—	—
G	—	Precipitate Previous step repeated until dissolved N is constant (0.100 mg. per cc. at 25°C).	—	—	—	—
H Active Protein	700 mg.	Precipitate	17	17	6	6

of value. If the native protein present in the press juice exerts no ad-
sorptive action, this added activity should fail to sediment appreciably.
Rosenfeld's experiments do not take into account the possibility that the

TABLE 9

PROPERTIES OF THE POSTERIOR LOBE PROTEIN

Activity, units per mg.	
Pressor	16.6
Oxytocic	16.6
Antidiuretic	16.4
Elementary analysis, per cent	
Carbon	48.64
Hydrogen	6.63
Nitrogen	16.32
Amino nitrogen	0.054
Phosphorus	0.027
Sulfur	4.89
Chlorine	0.02
Ash	0.58
Oxygen (by difference)	22.89
Sulfur distribution	
Cysteine	0
Cystine	4.3
Methionine	?
Sulfate	0.1–0.4
Molecular weight (ultracentrifuge)	30,000
Isoelectric point (electrophoresis)	4.8

brief heat treatment of the press juice might alter the adsorptive properties of the native protein through partial denaturation, thereby liberating a portion of the adsorbed activity.

The current work of van Dyke and his associates places the concept of a unitary hormone in the forefront of present biochemical research on the pituitary problem. Since the evidence obtained by these investigators is of great significance in our evaluation of the multiple and unitary hormone theories, a detailed description of their results is in order.[68]

The purification procedure used by van Dyke and his collaborators is presented schematically in TABLE 8. From 1 kg. of posterior lobes 700 mg. of the purified product are obtained; the product is an amorphous protein. It has a pressor, oxytocic and antidiuretic activity of 17 units per mg. and contains, therefore, about 6 per cent of the activity present in the original material. It is practically devoid of melanosome-dispersing activity.

The composition and properties of the isolated protein are tabulated in TABLE 9. The molecular weight, calculated from the amino nitrogen value, assuming one amino group per molecule, is 26,000. This agrees fairly well with the figure calculated from ultracentrifuge data. The

[68] Van Dyke, H. B., Chow, B. F., Greep, R. O., & Rothen, A. Jour. Pharmacol. 74: 190. 1942.

cystine sulfur value is very high and corresponds to a content of about 16 per cent cystine or 20 cystine molecules in a protein of 30,000 molecular weight.

The active protein is only 25 per cent inactivated by the action of crystalline pepsin at pH 3 and 37° C. for 6 days. Under these conditions 36 per cent of the protein is digested as calculated from the amino nitrogen increase. In similar experiments with trypsin and chymotrypsin pH 7.6 for 43 hours the activity is almost completely destroyed. With trypsin 70 per cent of the protein is digested; with chymotrypsin, 95 per cent is digested. Only oxytocic activity was tested in these experiments.

The assay of the protein by various procedures is given in TABLE 10.

TABLE 10
ASSAY OF THE PROTEIN FROM THE POSTERIOR LOBE

Method of assay	Average activity.* Units per mg. protein	Standard error*
Isolated guinea pig uterus	17.5	0.9
Blood pressure of fowl	15.7	0.6
Blood pressure of cat	15.7	—
Blood pressure of dog	17.5	0 6
Diuresis inhibition in rat	16.4	—
Melanosome dispersion in frog	0.008	—

*Van Dyke and coworkers express their potencies in micrograms of nitrogen equivalent to 1 U.S.P. unit. To simplify comparison with previous work, these figures have been recalculated on the basis of units per mg. of protein.

It will be noted that the various activities are present in equal amounts in the purified product, a condition which is one of the requirements of the unitary hormone. Consequently, the isolated protein was carefully examined to determine its homogeneity and to demonstrate as convincingly as possible whether or not the pressor, oxytocic and antidiuretic activities are integral parts of the protein molecule, since it was realized by van Dyke and his associates that the purity and inherent activity of the isolated protein must be established beyond all doubt if it is to be considered the "mother-molecule" and probable precursor of the separate posterior lobe hormones.

The group of workers at the Squibb Institute has gone to great lengths to provide the required proof. Their results appear to indicate that: (1) The protein possesses a constant ratio of pressor, oxytocic and antidiuretic activities (1:1:1). (2) The ratio of activities in the protein is identical to that existing in the untreated gland. (3) The protein exhibits a solubility curve typical of a pure substance. (4) The protein

~ediments in the ultracentrifuge as a pure protein. (5) Assay of various fractions from solubility, electrophoresis and ultracentrifuge tests gives no indication of the presence of components of greater or lesser potency than the protein itself. Though van Dyke and his associates believe that the results of these experiments are strongly in favor of the homogeneity of the product and the presence of the activities as integral parts of the protein, they nevertheless suggest that the results obtained so far are open to two interpretations: (1) The protein, although pure to the extent that present physicochemical methods permit such a conclusion, is pharmacologically active because of the adsorption of the highly active separate principles. (2) The protein, in part composed of active principles which can be separated from it, is elaborated and stored by the *pars neuralis.*

It is apparent that the first of the above alternatives must be ruled out completely before the second explanation can be accepted. Consequently, it may be profitable to ascertain what has been established by the researches of van Dyke and coworkers and to suggest any additional experiments which might aid in the eventual solution of this problem.

Solubility Data

Constant solubility (0.1 mg. nitrogen per ml.) is obtained with the protein in a solvent consisting of 0.5 M acetate buffer, pH 3.90, to which 6.5 gm. of NaCl are added for each 100 cc. In the same solvent constant solubility as low as 0.08 mg. nitrogen per ml. was also found with some batches of the isolated protein. No evidence for the presence of more than one component could be found in the solubility curve either before saturation with the active product or after 20 times the saturating concentration was in suspension. Over the range of the entire solubility curve the dissolved pressor and oxytocic activities were found to be equal and constant. The pressor and oxytocic potency of a sample of the pure protein was found to be unchanged after it was washed three times with the solvent used in the solubility tests, even though 54 per cent of the total activity was removed in the process. Furthermore, the supernatant fluid from a suspension of the protein in the same solvent, under conditions in which the solid phase was present far in excess of the saturation concentrations, showed the same pressor and oxytocic potency as the protein itself.

Solubility tests have been made only in a solvent of pH 3.90. As this solvent is identical in pH and composition with that used in attaining constant solubility in the final steps of the isolation procedure (see TABLE

8), the subsequent determination of solubility under the same conditions would be expected to furnish no additional information regarding purity. In fact, use of the same solvent in the isolation and in the purity tests would maintain any conditions responsible for the possible association of adsorbent and adsorbate if such should be the case. In this connection it may be significant that electrical inhomogeneity was found in electrophoresis experiments in which the pH of the medium was varied between 3.4 and 6.1, whereas no evidence of inhomogeneity was observed in solubility and ultracentrifuge experiments when the pH was allowed to vary only from pH 3.3 to 3.9. When sufficient amounts of the protein are available to permit such experiments, solubility curves, together with assays for each of the activities at different points in the solubility curve should obviously be carried out at several pH values, and, if possible, in solvents of different composition. If conditions could be found in which the active material is dissociated from the protein, it should be possible to isolate the inactive protein. Suitable amounts of the separated principles could then be added to the inactive protein and experiments could be carried out to determine whether or not a product of constant solubility and activity is again obtained under the conditions used in the original isolation procedure. These data should provide information as to whether the protein is a pure, active substance which is fragmented by the treatments employed, or a protein whose activity is simply adsorbed or eluted depending upon the conditions of the solvent. If dissociation of the protein and activity cannot be accomplished under any of the conditions employed, it would be desirable to study the solubility curve and activity at different points on the curve, after adding known amounts of the separated active principles to the active protein. If activity is actually adsorbed then it might be possible, for instance, to isolate a product having a pressor:oxytocic ratio greater than unity by precipitating the protein in a solution which has been enriched with respect to the pressor principle through the addition of known amounts of a purified pressor preparation.

Ultracentrifuge Data

In the ultracentrifuge, in the pH range 3.30 to 3.84, the isolated product appears to be a single, homogeneous protein with a sedimentation constant of 2.61 to 2.80 S*. From this figure, and an assumed specific volume of 0.749, a molecular weight of approximately 30,000 is calculated. Homogeneity was shown by the symmetry of the sedimentation curves and by the absence of significant displacement of the base line.

* All sedimentation constants are expressed in Svedberg units, denoted S, and equal to 10^{-13} sec.

The distribution of activity between the sedimented protein fraction and the supernatant fluid was determined after ultracentrifuging. Almost 100 per cent of the pressor and oxytocic activity was found to sediment with the protein. The authors concluded from these data that both pressor and oxytocic activities were associated with the protein and that there is no evidence favoring the presence of additional small, nonprotein components with high biological activity.

For the reasons presented in the discussion of the solubility data, ultracentrifuge experiments with the active protein should be tried at widely different pH values. Similarly, the behavior of the active protein in the presence of known amounts of the separate principles should also be investigated in the ultracentrifuge under the conditions reported for the protein alone. In the latter experiments it should be possible to demonstrate whether or not the added, low molecular weight principles sediment with the protein under these conditions.

Electrophoresis Data

The isoelectric point of the protein was estimated to be pH 4.8 by determining the electrophoretic mobility over a pH range from 3.4 to 6.2. At pH 3.41 to 3.47 the electrophoretic patterns showed the presence of one main component, but a second component, present in very small amount, also appeared. At all other pH values studied, electrical inhomogeneity was found but no second component could be discerned.

After electrophoresis of solutions of the protein at pH 3.4 to 3.5 the potencies of the different fractions (main component, "portein-free solution," and the minor component) were indistinguishable on the basis of nitrogen. Accordingly, it was suggested that the minor component either derived from or was closely related to the main protein component.

Interpretation of these results is difficult. Electrophoretic patterns obtained with the isolated protein give definite evidence of inhomogeneity, yet assays performed upon various fractions of the electrophoresis cell apparently fail to reveal the presence of substances of greater or lesser potency than the protein. Inasmuch as electrical inhomogeneity exists it is rather surprising that all of the components of the mixture possess the same pharmacological potency. Experiments should be devised in which separation of the components is accomplished under conditions such that sufficient active material is present in each of the fractions to afford complete and accurate assays for all of the activities. In this connection it will be recalled that previous electrophoresis experiments on untreated posterior lobe press juice,[10] performed under conditions which differed from those of van Dyke and his associates,

gave evidence in favor of the presence of separate pressor and oxytocic hormones. In the electrophoresis of the active protein, therefore, there is a possibility that the electrical inhomogeneity observed at certain pH values may be an indication of the separation of protein and adsorbed active principles.

The recovery of all activities should be investigated in electrophoresis experiments to show that preferential destruction of certain activities has not occurred. and to show that appreciable amounts of any rapidly moving pressor and oxytocic hormones present have not migrated beyond the limits of the electrophoresis cell. Experiments in which alternating current of the same potential used in the electrophoresis is employed might be used to ascertain the effect of the current alone upon the active protein. The homogeneity of material treated in this manner could then be studied by means of solubility and ultracentrifuge tests.

Since from the standpoint of the unitary hypothesis so much depends upon the constancy of the ratio between the activities, it must be emphasized that assays for all of the activities should be carried out upon the various fractions obtained during solubility, electrophoresis and ultracentrifuge experiments, and in any other experiments in which the separation of activities is a possibility.

Action of Cysteine Upon the Active Protein

A significant difference between the separate pressor and oxytocic principles and the active protein is found in the behavior of these substances toward the reducing action of cysteine and thioglycollic acid. Sealock and du Vigneaud[61] found that cysteine reduction of the pressor and oxytocic hormones caused no loss of pharmacological activity. On the other hand. van Dyke and coworkers find that treatment of the protein with thioglycollic acid at pH 7.5 completely destroys both pressor and oxytocic activity in 2 to 5 minutes. With cysteine instead of thioglycollic acid, activity is destroyed but at a slightly slower rate. A 44 per cent loss of activity was found after 2 minutes and after 5 minutes approximately 80 per cent loss occurred.

The observations that cysteine and thioglycollic acid destroy the activity of the protein but cysteine causes no inactivation of the separate principles, may seem to indicate rather strongly that the active protein is not merely an inactive substance with adsorbed active principles. The importance of these results warrants careful repetition under rigorously controlled conditions. It would be desirable to know whether the results of Sealock and du Vigneaud can be confirmed when the separate principles are subjected to cysteine reduction under the conditions used

by van Dyke and associates. If confirmation of the results with the separate principles is secured, while treatment of the active protein under exactly the same conditions still causes inactivation, it will be necessary to repeat the procedure on a mixture of the protein and the separate principles to determine whether the presence of protein during the reduction may be responsible for the difference between the behavior of the two preparations. It should be pointed out that the results as reported by Sealock and du Vigneaud and by van Dyke and associates are not necessarily incompatible. Reduction of the purified individual principles may be quite different from the reduction of the protein complex reported by van Dyke and his collaborators.

SUMMARY

The present status of the posterior pituitary hormone problem may be briefly summarized as follows. Separate pressor and oxytocic hormones can be isolated from the posterior pituitary lobe. These hormones have not yet been obtained in the pure state but all evidence points to the fact that the highly potent pressor and oxytocic preparations now available represent fairly pure preparations of separate molecules which may be similar chemically.

A protein which possesses pressor, oxytocic and antiduiretic activity has also been isolated from the posterior lobe. Moreover, evidence has been presented which indicates that this protein is pure and that the various activities are inherent properties of the molecule. Although this evidence is in favor of the tentative conclusions reached, it is believed that the question should be investigated further before the protein can be accepted unqualifiedly as a hormone of the posterior lobe possessing multiple activities.

THE CHEMISTRY OF THYLAKENTRIN, THE FOLLICLE-STIMULATING HORMONE OF THE ANTERIOR PITUITARY

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INTRODUCTION

Significant progress has been made in the chemical characterization of three anterior pituitary hormones which contribute to the regulation of the gonads. They are: metakentrin,[1-4] a hormone which stimulates the interstitial cells of the ovaries or testes and causes the formation of *corpora lutea* from preformed graafian follicles; thylakentrin,[5-7] a gametogenic hormone which causes the growth of graafian follicles preparatory to the release of ova from the female gonad and stimulates the sperm-forming tissue of the testes; and lactogenic hormone,[8,9] recently shown to have an important role in bringing about *corpus luteum* function[10-12] in hypophysectomized rats. Other gonadotrophic principles such as the antagonist[13] and synergist[14] have been postulated. The present data[15-18] favor the view, however, that synergism (or augmentation) is due to the combined action of thylakentrin and metakentrin on the ovary and that the antagonist[19-21] is identical with metakentrin. Since the chemistry of metakentrin (ICSH, LH), and lactogenic hormone, will be presented in other communications, these hormones will

1 Fevold, H. L., Lee, M., Hisaw, F. L. & Cohn, E. J. Endocrinology 26: 999. 1940.
2 Li, C. H., Simpson, M. E., & Evans, H. M. Endocrinology 27: 803. 1940.
3 Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B., & Chow, B. F. Science 92: 178. 1940.
4 Cohn, H. C. & van Dyke, H. B. Science 93: 61. 1941.
5 Fraenkel-Conrat, H., Simpson, M. E. & Evans, H. M. Annales de la Facultad de Medicina, Montevideo. XXV. 1940.
6 Greep, R. O., van Dyke, H. B., & Chow, B. F. Jour. Biol. Chem. 133: 289. 1940.
7 McCullagh, D. R. & Bowman, W. E. Endocrinology 27: 525. 1940.
8 White, A., Catchpole, H. R. & Long, C. N. H. Science 86: 82. 1937.
9 Li, C. H., Lyons, W. E. & Evans, H. M. Jour. Gen. Physiol. 24: 303. 1941.
10 Evans, H. M., Simpson, M. E. & Turpeinen, K. Anat. Rec. Suppl. 3. 70: 26. 1938.
11 Evans, H. M., Simpson, M. E., Lyons, W. R. & Turpeinen, K. Endocrinology 28: 933. 1941.
12 Evans, H. M., Simpson, M. E. & Lyons, W. R. Proc. Soc. Exper. Biol. and Med. 46: 586. 1941.
13 Evans, H. M., Korpi, K., Pencharz, R. F. & Simpson, M. E. Univ. of Calif. Publ. in Anatomy 1: 237. 1936.
14 Evans, H. M., Pencharz, R. F. & Simpson, M. E. Endocrinology 18: 601. 1934.
15 Fevold, H. L. & Hisaw, F. L. Am. Jour. Physiol. 109: 655. 1934.
16 Jensen, H., Simpson, M. E., Tolksdorf, S. & Evans, H. M. Endocrinology 25: 67. 1939.
17 Fraenkel-Conrat, H., Li, C. H., Simpson, M. E. & Evans, H. M. Endocrinology 27: 793. 1940.
18 Greep, R. O., van Dyke, H. B., & Chow, B. F. Am. Jour. Physiol. 133: 303. 1941.
19 Fevold, H. L. & Fiske, V. M. Endocrinology 24: 823. 1939.
20 Jensen, H., Simpson, M. E., Tolksdorf, S. & Evans, H. M. Endocrinology 25: 57. 1939.
21 Greep, R. O., van Dyke, H. B., & Chow, B. F. Am. Jour. Physiol. 133: 303. 1941.

not be considered in this report. The present discussion will therefore be limited chiefly to the follicle-stimulating hormone, thylakentrin.

METHODS OF ASSAY

OVARIES. Thylakentrin induces the growth of a large number of graafian follicles resulting in gross enlargement of the ovaries. This effect, usually measured by changes in weight, less commonly by histological means, has formed the principal basis of assay. No other single gonadotrophic agent is known to increase significantly the weight of the ovary.

In order to avoid the complication caused by the secretion of hormone by the test animals' own pituitaries, it is necessary to use animals in which this gland has been surgically removed (hypophysectomy). Recent work[20][21] dictates that not only the ovaries of such animals treated with thylakentrin must show the absence of *corpora lutea* to indicate a purely follicle-stimulating effect, but also that the interstitial tissue must be shown to be in an atrophic condition.

UTERUS. Estrogenic development of the female reproductive tract is a constant feature of the sexual precocity induced in immature animals by certain gonad-stimulating preparations. Some workers[22] have utilized the increase in the weight of the uterus as an indirect method of measuring ovarian stimulation presumed to be follicle-stimulating in effect. While this method will undoubtedly yield quantitative data, the basic assumption that the response is a measure of any specific gonadotrophic effect can be seriously questioned. The mechanism of estrogen secretion by the ovary is itself an unsolved problem. As we shall see later, thylakentrin alone appears not to be able to cause estrogen secretion; therefore, follicle growth can be obtained independent of any uterine growth.

VAGINA. Some authors[23] have recommended the production of vaginal oestrus as a basis for the assay of thylakentrin. The objections mentioned in the discussion of assay by determining the extent of uterine hypertrophy apply with equal force to vaginal changes which also appear not to afford a reliable method of assay.

OVULATION IN THE RABBIT. This method of assay is not specific but might have quantitative value provided that biological purity had been demonstrated.

TESTES. The gonads of hypophysectomized male rats show an increase in weight due solely to stimulation of the sperm-forming tissue.

22 Levin, T. & Tyndale, H. H.   Endocrinology 21: 619.   1937.
23 Witschi, E.   Endocrinology 27: 437.   1940.

This reaction is not accompanied by any testicular secretion of androgens; hence the secondary sexual organs such as the prostate and seminal vesicles are not benefited. The weight of the testes bears a quantitative relationship to the dose of thylakentrin administered within a restricted range. This method[24] can be used only when metakentrin, which also causes an increase in the weight of the testes (presumably by a secondary reaction to the androgen produced), is absent.

SOURCES

The gonadotrophic hormone, thylakentrin, may be obtained from pituitary glands or from the urine of normal, menopausal or gonadectomized human beings. This hormone in the urine can be concentrated by precipitation with tannic acid,[25,26] alcohol[27] or by other means without much loss of activity. By further fractionation of the hormone "tannate," a preparation effective in producing follicle stimulation, but essentially devoid of luteinizing ability, can be obtained. So far little chemical data have been accumulated on the follicle-stimulating hormone from urine.

Preparation of the Thylakentrin from Pituitary Glands

Both human and horse pituitary glands are relatively rich in thylakentrin but poor in metakentrin. It would appear that from such raw material, potent thylakentrin fractions free from metakentrin are likely to be obtained. But the practical difficulty of securing adequate supplies of human or equine glands has usually forced investigators to turn to the less suitable but more plentiful sheep and hog glands.

Ever since Fevold, Hisaw, and Leonard[28] asserted that they were able to separate the luteinizing from the follicle-stimulating hormone by chemical fractionation, many investigators have attempted to reach the same goal by improved methods. As a whole, the earlier workers used acetone-dried pituitary powder as the starting material. As knowledge of the chemistry of proteins became more general, however, fresh pituitary glands instead of dried powders were preferred, for acetone is known to denature and alter proteins.

Common procedures used to effect the separation of the two gonadotrophic hormones by different workers may be summarized as follows:

  1. Evans[29] and his collaborators utilized the greater solubility of

[24] Greep, R. O., van Dyke, H. B. & Chow, B. F. Anat. Rec. 78: 88. 1940.
[25] Hellbaum, A. A., Fevold, H. L. & Hisaw, F. L. Proc. Soc. Exp. Med. 32: 1566. 1934-35.
[26] Levin, L. Endocrinology 28: 378. 1941.
[27] McCullagh, D. R. & Bowman, W. E. Endocrinology 27: 525. 1940.
[28] Fevold, H. L., Hisaw, F. L., & Leonard, S. L. Am. Jour. Physiol. 97: 291. 1931.
[29] Evans, H. M., Korpi, K., Penchars, R. F. & Simpson, M. E. Univ. of Calif. Publ. in Anatomy 1: 237. 1936.

thylakentrin in ammonium sulfate solution. In his recent paper with Fraenkel-Conrat,[?] an improved scheme has been published. Other investigators[?] also utilized the same technique to effect a separation.

2. A method of Fevold, like that of Evans, depended on the greater solubility of thylakentrin in a certain concentration of ammonium sulfate or acetone. He made use of his discovery that metakentrin shows a minimum solubility at pH 4.2.

3. Wallen-Lawrence[13] separated metakentrin from thylakentrin by precipitation with alcohol at −6° C. The precipitate at 40 per cent alcohol was predominantly luteinizing, while that at 55 per cent alcohol was follicle-stimulating.

4. In our laboratory[?] we found that hog metakentrin is extremely insoluble in a solvent consisting of M 4 acetate buffer at pH 4.4 and 20.5 per cent Na_2SO_4, whereas thylakentrin is very soluble in such a solvent.

BIOLOGICAL ACTIVITY OF FOLLICLE-STIMULATING EXTRACTS

It is impossible to compare quantitatively the biological potency of the products isolated in the different laboratories because of differences in assay technique. Furthermore, a comparison of potency is no indication of relative chemical purity because the extracts have often been made from glands of different species of animals. But a qualitative difference needs emphasis: the injection, in relatively large doses, of thylakentrin preparations made by workers of other laboratories causes uterine hypertrophy, whereas our purified preparations fail to show any such stimulation. This can be seen from TABLE 1. Although as little as 3 micrograms solid of the Fraenkel-Conrat preparation gave follicular stimulation but no estrous uteri, estrus was definitely produced in 50 per cent of the rats if the dose was increased to 18 micrograms of solid. Similarly, Fevold's preparation of thylakentrin increased uterine weight. Greep, van Dyke and Chow[34] prepared extracts of thylakentrin from hog pituitary glands. Although 62.5 micrograms of their extract stimulated the growth of follicles in hypophysectomized rats, ten times such a dose did not cause estrogen secretion. To test more rigidly the question whether thylakentrin, when essentially free from metakentrin, causes estrogen secretion, these authors injected 1562 micrograms of thylakentrin into hypophysectomized rats over a period of ten days. None of the treated rats was in estrus.

[40] Fraenkel-Conrat, H., Simpson, M. E. & Evans, H. M. Annales de la Facutad de Medicina, Montevideo. XXV: 1940.
[1] Rinderknecht, H. & Williams, P. C. Jour. Endocrinology 1: 117. 1939.
[12] Jensen, H., Tolksdorf, S., & Bamman, F. Jour. Biol. Chem. 135: 791. 1940.
[13] Wallen-Lawrence, Z. Jour. Pharmacology & Exp. Therap. 51: 263. 1934.
[34] Greep, R. O., van Dyke, H. B. & Chow, B. F. Jour. Biol. Chem. 133: 289. 1940.

TABLE 1

THE UTERINE RESPONSE OF HYPOPHYSECTOMIZED RATS RECEIVING PURIFIED THYLAKENTRIN (FSH) PREPARED FROM DIFFERENT LABORATORIES.

Method of preparation	Dose in micrograms solid		Number of rats	Ovaries		Uterus
				Weight in mg.	Histology	
Fraenkel-Conrat, Simpson & Evans[a]	0		100	11	O	0*
	3	(1 unit)	9	14	F	0*
	15		8	30	F	1*
	18		8	39	F	4*
Fevold[b]	0		—	6	—	—
	20		—	10	—	0†
	40		—	16	—	0†
	80		—	24	—	50%†
	200		—	31	—	70%†
	500		—	35	—	170%†
Greep, van Dyke & Chow[c]	0		28	8.63	O	0*
	62.5		4	12.47	F	0*
	625.0		5	16.28	F	0*
	1562.0	(10 days)	6	15.1	F	0*
	0	(10 days)	6	5.7	O	0*

* Number of rats with estrous uteri.
† Percentage increase in average uterine weight.
[a] Fraenkel-Conrat, H. L., Simpson, M. E. & Evans, H. M.   Proc. Soc. Exp. Biol. & Med. 45: 627. 1940.
[b] Fevold, H. L.   Endocrinology 28: 33. 1941.
[c] Greep, R. O., van Dyke, H. B. & Chow, B. F.   Endocrinology 30: 635. 1942.

Although Fraenkel-Conrat and Fevold prepared pituitary extracts from sheep glands, and Greep, van Dyke and Chow obtained theirs from hog glands, it seems probable that the question of estrogen secretion of rats receiving thylakentrin extract is more dependent on the degree of contamination by metakentrin than on a difference in the animal source of the extract.   The maximum amount of metakentrin present in our thylakentrin preparation as contaminant was determined in two ways:

1. THE PROSTATE METHOD.   Greep, van Dyke, and Chow[35] found that as little as 2 micrograms of pure metakentrin nitrogen isolated from hog pituitary glands can bring about a significant increase in the weight of the anterior prostate of hypophysectomized rats.   The weight of the anterior prostate was not significantly increased by 360 micrograms of nitrogen of our best preparation of thylakentrin.   It might be added also that as little as 2 to 5 micrograms nitrogen of our thylakentrin preparation could bring about a significant increase in the testicular weight.   Since the metakentrin nitrogen in the total of 360 micrograms must be

[35] Greep, R. O., van Dyke, H. B., & Chow, B. F.   Proc. Soc. Exp. Biol. and Med. 46: 644. 1941.

less than 2 micrograms, the contaminating hormone must be less than 0.5 per cent.

2. THE IMMUNOLOGICAL METHOD. When rabbits were immunized against pure hog metakentrin, antibodies[36] may be detected in the sera of the immunized animals by the precipitin reaction. Such antisera will react with as little as one microgram of hog metakentrin protein, but will not react with metakentrin from other species of animals or with other pituitary hormones from hog glands. Once the immunological specificity of the anti-hog-metakentrin sera is established it is possible to detect the amount of metakentrin present in various fractions. It was found that 2500 micrograms of our hog thylakentrin extract failed to react with anti-metakentrin rabbit sera. Thus the thylakentrin extract contained less than 0.04 per cent of metakentrin. Tests for other pituitary hormones such as the lactogenic, thyrotrophic, chromatophorotropic, posterior lobe, and adrenotrophic hormones gave uniformly negative results. Therefore, our preparation of thylakentrin may be considered biologically pure in the sense that no other hormones have been found by biological assay to be present in the thylakentrin extract.

PHYSICO-CHEMICAL PROPERTIES
Effect of Enzymes

The action of proteolytic enzymes on pituitary gonadotrophic hormones has been studied by several groups of investigators. Tryptic digestion of pituitary extracts has yielded divergent results.

Chen and van Dyke[37] found that tryptic digestion abolishes most of the luteinizing action of extracts of sheep or horse pituitary, but leaves the follicle-stimulating factor unimpaired. Large doses of the digested extracts were followed by the formation of lutein tissue in the ovaries of hypophysectomized immature rats. Similar results were independently reported by McShan and Meyer[38]. Both groups of authors used normal as well as hypophysectomized rats and studied the microscopic appearance of the ovaries. The destructive effects of crystalline trypsin on thylakentrin were not recognized by McShan and Meyer. In neither set of observations was the extent of digestion determined. The differential lability of thylakentrin and metakentrin to tryptic action was not confirmed by Abramowitz and Hisaw[39]. They found that crystalline trypsin destroys both gonadotrophic hormones at about the same rate. Their conclusions were based on the changes in weight of the ovaries or

36 Chow, B. F.   Endocrinology 30: 657.   1942.
37 Chen, G., & Van Dyke, H. B.   Proc. Soc. Exp. Biol. & Med. 40: 172.   1939.
38 McShan, W. H. & Meyer, R. K.   Jour. Biol. Chem. 126: 361.   1938.
39 Abramowitz, A. A. & Hisaw, F. L.   Endocrinology 25: 633.   1939.

seminal vesicles of normal rats. Lacking histological studies and determinations of extent of digestion, their data do not appear to justify the belief that neither crystalline trypsin nor chymotrypsin destroys luteinizing hormone more rapidly than follicle-stimulating hormone.

Chow, Greep and van Dyke[40] carefully re-examined the effects of digestion by proteolytic enzymes (including crude and crystalline trypsin, on the gonadotrophic activities of anterior pituitary by comparing the actions of digested and incubated control extracts in hypophysectomized immature female and male rats. We found that the percentage of protein digested was often of decisive importance in determining destruction or survival of the hormones. Thus, if the tryptic digestion of the crude hog pituitary extract which contains both metakentrin and thylakentrin had proceeded to a moderate extent (less than 48 per cent), follicular growth was stimulated but the luteinizing action was absent. If digestion was carried to 61 to 75 per cent, both follicle-stimulating and luteinizing activities were abolished. It seemed reasonable to us, therefore, that the discrepancy between the results of Abramowitz and Hisaw, and those of other authors could be explained on the basis of differences in the degree of digestion.

In a recent paper, McShan and Meyer[41] described a reproducible method by which a crude extract, containing both follicle-stimulating and luteinizing activities, can be converted to a product having follicle-stimulating activity only. The degree of digestion was followed by the increase of amino-nitrogen content. Their results, in agreement with ours, showed that although the luteinizing activity can be easily destroyed by tryptic digestion even to a moderate extent, the survival of the follicle-stimulating activity is dependent upon the degree to which the gonadotrophic extracts are digested by trypsin. The same authors further showed that ptyalin or takadiastase destroys the biological activity of thylakentrin. Since these enzymes are assumed to attack carbohydrates but not proteins, it seems reasonable to conclude that carbohydrate also plays an important part in the biological activity. The carbohydrate is not separable from the protein by dialysis or by chemical fractionation, and it is assumed that it is united by chemical bonds. Thus thylakentrin is probably a glycoprotein. The proof of such a hypothesis lies in the eventual isolation of this hormone in a chemically pure form. Because of the importance of the carbohydrate group, attempts have been made to identify[42,43] and to correlate the carbohydrate con-

[40] Chow, B. F., Greep, R. O. & van Dyke, H. B. Jour. Endocrinology 1: 440. 1939.
[41] McShan, W. H. & Meyer, R. K. Jour. Biol. Chem. 135: 473. 1940.
[42] Fleisher, G., Schwenk, E. & Meyer, K. Nature 142: 835. 1938.
[43] Hartmann, M. & Benz, F. Nature 142: 115. 1938.

tent of crude extracts containing the follicle-stimulating hormone with biological activity. Evans[44] and others found that although the thylakentrin fraction of sheep pituitary extracts undoubtedly contained more carbohydrate than any other fraction of pituitary hormones, the determination of carbohydrate alone could not be taken as a measure of its follicle-stimulating potency. Therefore, they searched for a more specific chemical characterization of this gonadotrophic hormone and found that a high glucosamine as well as carbohydrate content are valuable guides in the purification of follicle-stimulating hormone. It must be emphasized here that the analyses were made on extracts of unknown chemical purity.

The Action of Other Chemical Agents on the Follicle-Stimulating Activity of Thylakentrin

EFFECT OF KETENE

Ketene is capable of reacting with all active hydrogens in the protein molecule, namely $-NH_2$, $-SH$, phenolic and alcoholic $-OH$. Among these groups, the amino group is most easily acetylated.

Li, Simpson, and Evans[45] subjected both thylakentrin and metakentrin to ketene treatment at pH 5. They found that acetylation for 5 minutes significantly decreased the potency of metakentrin. In contrast with this, reduction in the activity of thylakentrin did not occur in 5 minutes but did occur after 30 minutes of acetylation. The authors concluded from these experiments that the physiological activity of both thylakentrin and metakentrin is dependent on the free amino groups. They assumed that during acetylation under their experimental conditions (30 minutes at pH 5.7), ketene did not react with the hydroxyl group in the carbohydrate part of the molecule, because Neuberger[46] found ketene unable to acetylate the hydroxyl groups in carbohydrate resulting from the hydrolysis of egg albumin for as long as 18 hours at pH 6.0. They further assumed that phenolic groups were not attacked, because Stern and White[47] have shown that at least 45 minutes are necessary to cause appreciable acetylation of the phenolic groups of insulin. Such an assumption may not be entirely valid, because it is conceivable that the rate of reaction between the ketene and the phenolic groups of different proteins may differ. Such assumptions would not have been necessary if enough material were available for the determination of the ratio of the molar fraction of acetyl groups introduced to that of amino

[44] Evans, H. M., Fraenkel-Conrat, H., Simpson, M. E. & Li, C. H. Science 89: 249. 1939.
[45] Li, C. H., Simpson, M. E. & Evans, H. M. Jour. Biol. Chem. 131: 259. 1939.
[46] Neuberger, A. Biochem. Jour. 32: 1445. 1938.
[47] Stern, K. G. & White, A. Jour. Biol. Chem. 122: 371. 1937–1938.

groups acetylated, and also if no decrease of tyrosine groups was found. The acetylation could not occur on the sulfydryl group because ". . . thio groups have not been found in these substances."

EFFECT OF REDUCING AGENTS

In 1935 Maxwell and Bischoff[48] made some chemical studies on the effect of reducing agents on impure pituitary gonadotrophic hormone. They found no detectable loss of activity upon short exposure of hormone to nascent hydrogen, sulfur dioxide, hydrogen sulfide, ferrous sulfate, or hydrogen cyanide.

Different results were obtained by Fraenkel-Conrat, Simpson, and Evans[49]. They used cysteine, which reduces only the disulfide linkage of the protein, and found that unfractionated gonadotrophic extracts or purified thylakentrin, when treated with cysteine at pH 7.7 for two days, were more than 95 per cent inactivated. They therefore concluded that $-S-S-$ linkages are important to biological activity. McShan and Meyer[41] confirmed the inactivation of thylakentrin by reduction with cysteine. Their preparations, made by the trypsin method, were inactivated by treatment with 6 to 40 times their weight of cysteine at room temperature for 48 hours.

Bischoff[50] reopened the question of the reduction of gonadotrophic hormones. He studied the action of both cysteine and cyanide upon the hypophysial gonadotrophic hormones. He allowed cysteine to react with the hormones for 12 or 48 hours, and cyanide for 1 or 24 hours. His results indicated that increasing amounts of cysteine and extension of the reaction time increased the inactivation, but that the pituitary hormones were able to withstand a short exposure (1 hour) to cyanide in alkaline solution. 24 hours of exposure produced some inactivation.

These three groups of workers confirm one another concerning the reduction of biological activity of thylakentrin when it is exposed to cysteine for a period of 24 hours. But Bischoff disagreed with the hypothesis, suggested by Fraenkel-Conrat and coworkers and endorsed by McShan and Meyer, that $-S-S-$ linkages are an essential part of the active group of this gonadotrophic hormone. He suggested that the inactivation produced by a large excess of reagent and prolonged reaction time might be ascribed to side reactions, but he did not suggest what the side reactions might be.

[48] Maxwell, L. C., & Bischoff, F. Jour. Biol. Chem. 112: 215. 1935-36.
[49] Fraenkel-Conrat, H., Simpson, M. E. & Evans, H. M. Jour. Biol. Chem. 130: 254. 1939.
[50] Bischoff, F. Jour. Biol. Chem. 134: 641. 1940.

Interpretations Suggested by Ultracentrifugation
or Electrophoresis

Ultracentrifugation or electrophoretic analysis has been helpful in the isolation of many biologically active proteins. The first attempt to concentrate anterior lobe and pituitary-like hormone in the castrate and menopause urine was made by Severinghaus, Levin and Chiles[51]. They found that the gonadotrophic hormone from the urine could be concentrated by ultracentrifugation at 150,000 to 200,000 g. for 4 to 6 hours, indicating a relatively high molecular weight for the hormone, but there appeared to be no marked purification.

Electrophoretic studies of anterior pituitary proteins have been made by Shipley, Stern and White[52]. The preparations used included crude pituitary gland extracts obtained with dilute alkali, glycerol, or saline. The number of the main boundaries detected during electrophoresis was rather small 2-4), and the bulk of the protein was inert. Therefore, electrophoretic analysis of crude extracts has not given useful information on the electrochemical properties of the thylakentrin.

In collaboration with Doctors Shedlovsky and Rothen of the Rockefeller Institute for Medical Research, we have made a few electrophoretic and ultracentrifugal analyses of our biologically pure thylakentrin extracts. Our results showed definitely that they were electrically inhomogeneous and ultracentrifugally polydisperse. Details of all our experiments will not be given, but one typical electrophoretic separation experiment is worth mentioning. Using an acetate buffer of pH = 5.0 and $\mu = 0.05$, we found that one of the protein components moved anodically, while the remaining components moved cathodically (FIGURE 1). Separation into fractions was made mechanically. The number of components expected in each fraction is given in FIGURE 1 and TABLE 2.
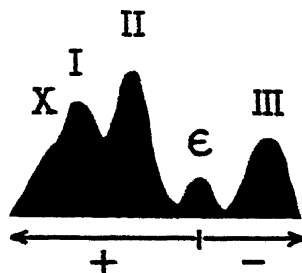
[51] Severinghaus, A. E., Levin, L. & Chiles, J. A.   Endocrinology 23: 285.   1938.
[52] Shipley, R. A., Stern, K. G., & White, A.   Jour. Exp. Med. 69: 785.   1939.

FIGURE 1.

TABLE 2

BIOLOGICAL ASSAY IN HYPOPHYSECTOMIZED RATS OF THE COMPONENTS OBTAINED BY ELECTROPHORETIC SEPARATION OF IMPURE SWINE THYLAKENTRIN.

Fraction number	Components present	Micro-gram N per dose	Num-ber of rats used	Weight of testes in mg.	Weight of anterior prostate in mg.
5	X + I	10	5	126.4	6.89
6	X + I (II)	10	5	110.4	7.48
7	III	10	3	178.6	6.57
10	III + (II)	10	5	178.6	6.76
Control		0	5	114.7	6.09

All fractions were analyzed for nitrogen and assayed in hypophysecto-mized male rats. The results of the assay are given in TABLE 2. It can be seen that the biological activity was concentrated in fractions 7 and 10. Fraction 7 contained only component III, but fraction 10 contained component III and a small amount of II. Since component II should also have been present in the inactive fraction 6, it seemed reasonable to conclude that component III was the hormone.

Similar separation experiments were made at pH 4.6. The active component moved as a cation. From the mobilities of the active component at pHs 5.0 and 4.6, we estimated the isoelectric point of hog thylakentrin to be about 4.8.

CONCLUSION AND SUMMARY

In the brief discussion of the chemistry of thylakentrin (FSH) the following points were emphasized:

1. METHODS OF BIOLOGICAL ASSAY. In evaluating different methods, emphasis was placed on the feasibility of quantitative estimation of the thylakentrin present in a given extract. If metakentrin is absent in the test extract, the increase of weight of either ovaries or testes may be used for quantitative assay. If metakentrin is also present, the interpretation of the biological result becomes difficult. Estrogen secretion apparently is not provoked by thylakentrin alone.

2. METHODS OF PURIFICATION. The chief accomplishment in the purification of thylakentrin has been the removal of other pituitary hormone proteins, particularly metakentrin (ICSH). The separation of these two gonadotrophic hormones can be effected by utilizing the relative insolubility of metakentrin in half saturated ammonium sulfate solution, in 40 per cent alcohol at −6°C., or in 20.5 per cent sodium sul-

fate buffered with acetate at pH 4.4.   Though the purified thylakentrin is not chemically pure, it can be obtained free from other pituitary hormones.   Our best preparations of thylakentrin did not stimulate estrogen secretion, even when large doses were given to hypophysectomized rats over a period of ten days.   It seems probable that the thylakentrin extracts which cause uterine hypertrophy are contaminated by metakentrin.   Estimation of metakentrin (hog) could be made either by the increase of the weight of the ventral prostate of the hypophysectomized rats or by the precipitin test with immune sera of rabbits immunized with pure hog metakentrin.

3. PHYSICO-CHEMICAL PROPERTIES.   Chen and van Dyke, and McShan and Meyer found that tryptic digestion abolishes most of the luteinizing hormone but leaves thylakentrin unimpaired.   Abramowitz and Hisaw, however, found that crystalline trypsin destroys both gonadotrophic hormones at about the same rate.   We found that these apparently divergent results could be harmonized, if the percentage of protein digested was taken into account.   In other words, if the crude hog pituitary extract was digested by trypsin to a moderate extent (less than 48 per cent), the digest could stimulate follicular growth, but the luteinizing action was absent.   If the digestion was carried out further (to 61–75 per cent) both thylakentrin and metakentrin, originally present in the extract, were destroyed.

Thylakentrin can also be inactivated by ptyalin or takadiastase.   The assumption is that these enzymes attack only carbohydrate.   Since both carbohydrate and protein play an important role in the biological activity, it is further assumed that thylakentrin is a glycoprotein.

The actions of chemical agents like ketene or cysteine have been studied.   Acetylation of thylakentrin with ketene for 30 minutes abolished its biological activity.   It was assumed that the destruction of the biological activity was due to the acetylation of the amino groups.   Cysteine likewise destroyed completely the activity of thylakentrin.   Whether this chemical action is one of reduction has not been conclusively proved.

Only a few studies of thylakentrin have been made using either electrophoresis or the ultracentrifuge.   The electrophoretic separation of thylakentrin in purified extracts of hog pituitary at different pHs suggested that the isoelectric point of the hormone is about 4.8.

THE LUTEINIZING HORMONE OF THE ANTERIOR LOBE OF THE PITUITARY BODY

By H. L. Fevold

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INTRODUCTION

Early indications of the existence of a pituitary luteinizing hormone were obtained by Evans and coworkers.[1] They reported that alkaline extracts of pituitary tissue produced excessive luteinization of the ovaries when injected into rats, while acidified extracts produced follicular growth with less luteinization. They ventured the opinion that the growth hormone might be the factor which produced luteinization. At approximately the same time, Ascheim and Zondek[2,3] reported the discovery of two gonadotrophic hormones in the urine of human beings. One of these stimulated the growth of follicles in the ovaries of rats and the second produced *corpora lutea*. Since it had been shown that the pituitary was essential for gonadal development[4], it was concluded that these substances had their origin in the pituitary. Subsequent work led to a great deal of discussion regarding the unity or duality of the pituitary gonadotropic complex, the question being complicated by the fact that the luteinizing hormone found by Ascheim and Zondek in the urine of pregnancy was not a pituitary hormone and should not have entered into the discussion. The luteinizing hormone of pregnancy urine is of placental origin, is not a pituitary hormone, and will not be considered in this paper.

Chemical fractionation of pituitary gonadotropic extracts resulting in the separation of a luteinizing hormone (LH) from a follicle-stimulating hormone (FSH) was reported in 1931[5]. Following that report, interest in the gonadotropic complex and in the existence of the luteinizing hormone as a separate individual was further stimulated. Further purification of the factor from other pituitary hormones together with physiological characterization of the purified preparations demonstrated that it carried out certain physiological functions which could not be carried out by the follicle-stimulating hormone or by any other pituitary

[1] Evans, H. M. & Simpson, M. E. Jour. Am. Med. Assn. **91:** 1337. 1928.
[2] Ascheim, S. & Zondek, B. Klin. Wchnschr. **6:** 1322. 1927.
[3] Zondek, B. Klin. Wchnschr. **8:** 245. 1930.
[4] Smith, P. E. Jour. Am. Med. Assn. **88:** 158. 1927.

hormone. Its place as a separate individual and as an important and indispensible factor in maintaining gonadal function was therefore gradually established.[5–15]

At the present time it appears to be well established that the luteinizing hormone of the pituitary is responsible for and carries out the following physiological functions. Together with the follicle-stimulating hormone it produces estrogen secretion from the ovarian follicles.[16–18] It takes part in the enlargement of the follicles which immediately precedes ovulation and which has been called preovulatory swelling.[19–21] It stimulates the development of luteal tissue and *corpora lutea*, which then secrete progesterone. The luteinizing hormone is not, however, the factor which maintains the *corpora lutea* in a functional, secretory state: a different luteotropic factor from the pituitary carries out that function.[22][23] In the male it acts on the interstitial cells of the testes, stimulating them to secrete the male hormone which then maintains the secondary sex glands and other secondary sex characters. It is therefore an essential substance for reproductive processes, both in male and female—as a luteinizing hormone in one and as an interstitial-cell-stimulating hormone in the other.

In addition to the evidence for the separate existence of the luteinizing hormone obtained by chemical purification and by physiological studies of the purified preparations, important confirmatory evidence for chemical differences in the two gonadotropic hormones has been found in studies of the action of enzymes on pituitary extracts. By subjecting such extracts to controlled digestion with trypsin or chymotrypsin it has been possible to destroy the luteinizing activity of the extracts and still retain the greater part of the follicle-stimulating activity. Conversely, it has been reported that pepsin destroys all of the follicle-stimulating activity with very little destruction of luteinizing activity if the

[5] Fevold, H. L., Hisaw, F. L. & Leonard, S. L.   Am. Jour. Physiol. 97: 291.   1931.
[6] Fevold, H. L., Hisaw, F. L., Hellbaum, A. & Hertz, R.   Am. Jour. Physiol. 104: 710.   1933.
[7] Fevold, H. L. & Hisaw, F. L.   Amer. Jour. Physiol. 109: 655.   1934.
[8] Wallen-Lawrence, Z.   Jour. Pharmacol. & Exp. Ther. 51: 263.   1934.
[9] Evans, H. M., Korpi, K., Simpson, M. E., Pencharz, R. I. & Wonder, D. H.   Univ. of Calif. Pub. in Anat. 1: 255.   1936.
[10] Greep, R. O., Fevold, H. L. & Hisaw, F. L.   Anat. Rec. 165: 261.   1936.
[11] Greep, R. & Fevold, H. L.   Endocrinology 31: 611.   1937.
[12] Fraenkel-Conrat, H., Li, C. H., Simpson, M. E. & Evans, H. M.   Endocrinology 27: 793.   1940.
[13] Greep, R. O.   Cold Spring Harbor Symposia Quant. Biol. 5: 136.   1937.
[14] Breneman, W. R.   Anat. Rec. 64 (Suppl. : 56.   1935.
[15] Breneman, W. R.   Anat. Rec. 64: 211.   1936.
[16] Fevold, H. L.   Anat. Rec. (Suppl. 2) 73: 19.   1939.
[17] Fevold, H. L.   Endocrinology 28: 33.   1941.
[18] Shedlovsky, T., Rothen, A., Greep, R. O., van Dyke, H. B. & Chow, B. F.   Science 92: 188.   1940.
[19] Foster, M. A. & Hisaw, F. L.   Anat. Rec. 62: 75.   1935.
[20] Foster, M. A., Foster, R. C. & Hisaw, F. L.   Endocrinology 21: 249.   1937.
[21] Hisaw, F. L., Greep, R. O. & Fevold, H. L.   Anat. Rec. Suppl. 64: 84.   1935.
[22] Evans, H. M., Simpson, M. E. & Turpeinen, K.   Anat. Rec. Suppl. 70: 26.   1938.
[23] Astwood, E. B.   Endocrinology 28: 309.   1941.

digestion is not allowed to proceed too far. Ptyalin (saliva) was found to destroy the FSH but not LH.[24—29]

The individuality and specificity of the two gonadotropic hormones is further demonstrated by some immunological experiments which have been reported. It has long been known that antigonadotropic substances are produced in animals when they are injected for long periods of time with gonadotropic extracts. Morever, it has been found that antisera produced against preparations which are rich in LH but poor in FSH will selectively neutralize the LH with very little effect on the FSH when injected together with an extract rich in both.[30]

A recent paper reports some further interesting experiments dealing with the production of antigonadotropic sera.[31] An unfractionated gonadotropic extract was subjected to tryptic digestion which digested the LH but not the FSH. This preparation was then used to produce antisera against FSH. When these antisera were injected with FSH alone, they completely neutralized the FSH effect. When the antisera were injected with an unfractionated extract containing FSH and LH, the FSH action was neutralized but no effect on LH activity was apparent. An antiserum produced by injecting both FSH and LH simultaneously, however, neutralized the effects of both when injected with them.

It is therefore possible to produce antisera which will neutralize either follicle-stimulating hormone or luteinizing hormone or both, depending on whether one or both were present in the extract which was injected to produce the antisera. Thus the two hormones would appear to be separate and distinct proteins.

OCCURRENCE OF THE LUTEINIZING HORMONE

The luteinizing hormone is present in the pituitaries of all animals which have been investigated. There is a species difference in the quantity of luteinizing hormone present, although the information is not too definite due to the fact that different assay methods have been used, and they have usually been carried out in the presence of the follicle-stimulating hormone, which may have some effect on the result. TABLE 1 lists

[24] McShan, W. H., & Meyer, R. K. Jour. Biol. Chem. 126: 361. 1938.
[25] McShan, W. H. & Meyer, R. K. Proc. Soc. Exp. Biol. Med. 40: 699. 1939.
[26] McShan, W. H. & Meyer, R. K. Proc. Soc. Exp. Biol. Med. 40: 701. 1939.
[27] Chen, G. & van Dyke, H. B. Proc. Soc. Exp. Biol. Med. 40: 172. 1939.
[28] Greep, R. Anat. Rec. (Suppl. 2) 73: 23. 1939.
[29] Chow, B. F., Greep, R. O. & van Dyke, H. B. Jour. of Endocrinology 1: 440. 1939.
[30] Rowlands, I. W. Jour. of Endocrinology 1: 172. 1939.
[31] Kupperman, H. S., Meyer, R. K. & McShan, W. H. Endocrinology 29: 525. 1941.

TABLE 1

LUTEINIZING HORMONE CONTENT OF THE PITUITARIES OF VARIOUS SPECIES

High	Moderate	Low
Cat	Rat	Beef
Baboon	Armadillo	Whale
Sheep	Guinea Pig	Horse
Opossum	Dog	Human
Cottontail Rabbit	Swine	
	Domestic Rabbit	

the pituitaries of various species in three groups, roughly classifying them with regard to their luteinizing hormone content.[32-35]

Information regarding the occurrence of luteinizing hormone in other tissues is meager. It has been reported to be present in blood but only in small amounts. It is apparently excreted in small amounts, usually together with follicle-stimulating hormone, in the urine of human beings throughout life.[36 37] The large amount of gonadotropic hormone (Prolan B) excreted in human pregnancy urine, as has already been pointed out, is of placental origin and is physiologically and chemically different from pituitary luteinizing hormone.

The gonadotropic preparations obtained from pregnant mare serum and which produce luteinization may or may not be composed of two substances, one of which is luteinizing hormone. Fractionation of mare serum into two gonadotropic substances has been reported[38,39] but more recently preparations of very high activity[40] and preparations which are electrophoretically homogeneous[41] have been prepared which are equivalent physiologically to the original crude serum.

There is, however, another piece of evidence which would seem to show that two gonadotropic hormones are present in pregnant mare serum.[31] This comes from immunological data dealing with the production of antisera. It has been reported that antisera produced against sheep pituitary FSH will inhibit the follicle-stimulating activity of pregnant mare serum when injected with it but has no effect on the luteinizing action. If this be substantiated, it would seem to prove the presence of a separate luteinizing protein also in pregnant mare serum. At least

32 Hill, R. T. Jour. Physiol. 83: 187. 1934.
33 Fevold, H. L. Endocrinology 24: 435. 1939.
34 Witschi, E. Endocrinology 27: 437. 1940.
35 West, E. & Fevold, H. L. Proc. Soc. Exp. Biol. Med. 44: 446. 1940.
36 Engle, E. T., Allen, Danforth & Doisy. "Sex and Internal Secretions." Second Edition. Chapter XVIII. Part II. Williams and Wilkins Co. Baltimore, Md.
37 Fevold, H. L. & Fiske, V. M. Endocrinology 24: 823. 1939.
38 Hellbaum, A. A. Proc. Am. Physiol. Soc. 119: 331. 1937.
39 Evans, H. M., Korpi, K., Simpson, M. E. & Pencharz, R. I. Univ. Calif. Pub. in Anat. 1: 275. 1936.
40 Cole, H. H., Pencharz, R. I. & Goss, H. Endocrinology 27: 548. 1940.
41 Li, C. H., Evans, H. M. & Wonder, D. Jour. Gen. Physiol. 23: 733. 1940.

it would prove that follicle stimulation and luteinization are produced by separate prosthetic groups which may or may not be attached to the same protein molecule.

ASSAY METHODS FOR THE LUTEINIZING HORMONE

A number of methods have been and are being used in determining the activity of LH preparations. All of these are biological and depend upon individual physiological properties of the hormone.

The first method used in quantitative work was based on the first known physiological property of the hormone, namely the production of *corpora lutea* in the ovaries of immature rats and the increased weight of the ovaries when LH is injected into immature female rats together with FSH. An amount of FSH is injected over a period of four days which produces approximately 100 per cent increase in weight over that of the ovaries of untreated rats. Various amounts of LH are then injected simultaneously with the same amount of FSH, and the additional increase due to the LH is determined. That amount of LH which produces an additional 100 per cent increase in the weight of the ovaries together with the production of *corpora lutea* is considered a unit.[42] This method has the obvious disadvantage of depending on the interaction of two substances and necessitates the use of FSH which is free of LH. This method can therefore be used only with purified materials.

Other methods, which have been used more often recently, depend on the physiological activity of the luteinizing hormone in the male. LH stimulates the interstitial elements of the testes to secrete androgenic hormone, which then acts on the secondary sex glands such as the prostate and the seminal vesicles. The increase in the weight of any one or of all the secondary sex glands may therefore be used as an indirect measure of the activity of the LH preparation.

The end point which we have used in our laboratory has been the weight increase in the seminal vesicles plus the coagulation gland.[33] Immature male rats, 21 to 22 days old at the beginning of the test, are injected twice daily for 4 days with varying amounts of the material to be tested. The volume of liquid is $\frac{1}{4}$ cc. per injection. The animals are then killed the morning of the fifth day; the seminal vesicles plus the coagulation gland are dissected and weighed.* From 50 to 100 per cent increase in the weights over those of seminal vesicles of control rats is taken as a unit. The seminal vesicles plus the coagulation glands of untreated rats 25 days old average 10.5 mg. with the limits 8.5 to 12.5

[42] Fevold, H. L. Cold Spring Harbor Symposia on Quant. Biol. **5:** 93. 1937.
* The structures removed also include a small portion of the dorsal prostate.

mg. The lowest dosage which increases the weights to 17 or 18 mg. is the unit. This would be a 100 per cent increase based on the lower limit and a 50 per cent increase based on the upper limit of untreated glands. The results obtained are consistent, and the method has been used successfully with rats derived from a Sprague-Dawley strain.

This method can be used to determine LH in unfractionated extracts if the injections are properly carried out.[43] If the injections are made subcutaneously, the true value of LH is not obtained in an unfractionated extract because FSH increases the responses to LH. But if an unfractionated extract is injected intraperitoneally, this augmentation does not take place when amounts ordinarily used for standardization are given, and the response is due primarily to the LH present. Pure LH, however, gives approximately the same result whether injected subcutaneously or intraperitoneally.

This method has been used in our laboratory with consistently reliable results, but the reports of other investigators indicate that the response to LH may not be alike in different strains of rats. Evans and coworkers find it difficult to produce any significant hypertrophy of the seminal vesicles with LH or with unfractionated extracts.[12] A preparation of LH furnished us by Doctor Evans—which in his animals (Long-Evans strain) produced very little seminal vesicle hypertrophy—quite readily produces as much as 600 per cent increase in seminal vesicle weight in our rats (Sprague-Dawley strain). Similarly, an unfractionated extract containing both FSH and LH produced maximal hypertrophy of the seminal vesicles in Sprague-Dawley rats but only about 30 per cent increase in Long-Evans rats. This is all the more surprising because Long-Evans rats do respond to implants of pituitary tissue.

Similarly, van Dyke and coworkers[44,45] found that in hypophysectomized rats of the Long-Evans strain the seminal vesicles do not respond markedly to LH injections but that the anterior lobe of the prostate serves as a better index of LH activity. Consequently they have used it as the end point. Their unit is described as an amount of material which produces a significant increase in the fresh weight of the anterior lobe of the prostate of rats hypophysectomized at 21 days of age. Injections are started 2 days after hypophysectomy and continued for 4 days, injecting once daily. Necropsy is performed 24 hours after the last injection.[15]

Evans and coworkers use hypophysectomized female rats to determine the activity of preparations of the luteinizing hormone.[12] The rats are

[13] Fevold, H. L.   Jour. Biol. Chem. 28: 83. 1939.
[14] Greep, R. O.   Proc. Am. Physiol. Soc. in press.
[15] Greep, R. O., van Dyke, H. B. & Chow, B. F.   Jour. Biol. Chem. 133: 289. 1940.

hypophysectomized when they are 26 to 28 days old, and the injections are started 6 to 8 days after the operation. At this time the interstitial cells of the ovaries have assumed a deficiency condition. The rats are then injected intraperitoneally, once daily for 3 days with the preparation to be tested. At the end of the injection period the ovaries are removed, sectioned, and examined microscopically. The amount of the preparation which is sufficient to bring the interstitial cells back to normal is defined as the unit.

An assay method which is said to be a direct measure of LH, rather than indirect as those enumerated above, has been reported by Witschi.[34] The animal used is the African weaver finch. The seasonal breeding plumage of the males of these birds is determined directly by the gonadotropic hormones of the pituitary and more particularly by the luteinizing hormone. The female and the male plumage during the nonbreeding season is white. When LH is injected in the breast muscles, a black spot or bar appears in the feathers 2 days after injection; and the minimum amount to produce the black bar is taken as a unit. This effect is believed to be very specific for the LH, and the presence of FSH has very little, if any, effect on the results.

The unfortunate situation exists at the present time that no two laboratories have used the same method for LH assay, and there are no comparisons of the results obtained by the various methods. Correlation of the quantitative data dealing with the luteinizing hormone is urgently needed. Comparison is made still more difficult by the fact that not only are the methods different but strains of rats which seem to be of different physiological reactivity are used. Much confusion and misunderstanding would be avoided, and research on the luteinizing hormone would be greatly facilitated if some agreement could be reached on a method for standardization. It would be most practical if the assay method devised could be used on intact animals, thus obviating operative procedures such as hypophysectomy.

EXTRACTION AND PURIFICATION OF THE LUTEINIZING HORMONE

The pituitary tissue which has been used for chemical investigations of the gonadotropic hormones has been obtained almost exclusively from sheep and swine. Cattle pituitaries are available but contain very little gonadotropic material. The pituitaries are removed at slaughter houses and are preserved in one of two ways. They are either frozen and stored, or are desiccated with acetone, ground and stored as powders.

Either of these procedures has been thought to retain the hormones in active form.

A report has recently been made of an investigation dealing with various methods of preserving pituitary tissue.[46] The pituitaries were desiccated with acetone, with alcohol, air dried, and also stored in the fresh frozen state. The subsequent biological assays showed a marked loss of activity when desiccation was effected with acetone or alcohol, but only a slight loss when the tissue was dried in air. Storage of sheep pituitary tissue in the frozen state, at $-10°$ C., resulted in no loss of gonadotropic activity after 80 days. From the results it appeared that autolysis of the tissue did not proceed very rapidly since no loss occurred on air drying. But the safest method for preservation and storage seems to be in the frozen condition.

Purification of the luteinizing hormone is effected by methods which are widely used in protein chemistry, namely: precipitation with salt, isoelectric precipitation, and by fractionation with aqueous acetone or alcohol. In the last two or three years a high degree of purification has been obtained in at least three laboratories. The preparations from two of these laboratories are reported to be pure proteins as determined by sedimentation in the ultracentrifuge, electrophoretic analysis and by solubility studies. The product obtained by the third group of workers appears not to be pure, as judged by these criteria, but seems to exceed those of the others in activity.

Van Dyke and coworkers used fresh hog pituitaries as their original material.[15 29 45] The method by which they obtain a pure protein preparation of the luteinizing hormone is as follows:

1. 1 kg. fresh hog pituitaries is extracted with 5 liters of cold 2 per cent NaCl.
2. pH is adjusted to 4.2–4.6 with N 70 HCl. Centrifuged.
3. Precipitate is washed twice with 2 per cent NaCl. 1.7 liters per washing.
4. Supernatants are combined and saturated with $(NH_4)_2SO_4$.
5. Precipitate is removed by filtration.
6. Precipitate is dialyzed in cold against distilled water until free of salt. Precipitate is removed and discarded.
7. pH is adjusted to 5.1. Precipitate is discarded.
8. Supernatants are brought to 50 per cent saturation with $(NH_4)_2SO_4$ at pH 4.2. Inactive precipitates are washed and discarded.
9. Solution is brought to 0.9 saturation with $(NH_4)_2SO_4$. Precipitate is collected by filtration and again dialyzed salt free.

[46] Kupperman, H. S., Elder, W. H. & Meyer, R. Endocrinology 29: 23. 1941.

10. One volume of 1M acetate buffer, pH 4.41 is added and two volumes of 41 per cent Na_2SO_4.

11. The precipitate is dissolved to a concentrated solution and the active material precipitated at $1\over3$ saturation $(NH_4)_2SO_4$, pH 7.3–7.4.

12. Precipitate is taken up in water and the active material precipitated by the addition of an equal volume of saturated ammonium sulfate, pH 7.3–7.4.

13. These last two steps are repeated 7 times and the final precipitate in a small volume of water is dialyzed salt free.

This material was found to contain 150 rat units per mg., 6.7 micrograms of the material being the minimal effective dose for the stimulation of the ventral prostate in hypophysectomized male rats.

Electrophoretic analysis of the material showed only one component to be present with a mobility of -3.85×10^{-5} at pH 4.58, -2.01×10^{-5} at pH 6.21 and 0.66×10^{-5} at pH 7.86. These measurements were made in acetate, cacodylate and diethyl barbiturate buffers of 0.05 ionic strength, and at temperatures near $0°$ C., the mobilities being corrected to $0°$.

The average sedimentation constant in a 1 per cent NaCl solution was $s_{20} = 5.39$ S.* The diffusion constant, determined in a cacodylate medium, was found to be $D_{20} = 5.9 \times 10^{-7}$. From these values the molecular weight was estimated to be approximately 90,000.

Solubility studies likewise indicated that this material was a pure protein because the solubility was independent of the amount of solid present in excess of saturation.

Evans and coworkers[47] used acetone-desiccated sheep pituitary tissue as their original material, and proceeded with the extraction and purification of the active principle in the following manner:

1. Three hundred grams of acetone-dried whole sheep pituitary powder are extracted twice with 40 per cent ethyl alcohol (4 liters followed by 2 liters).

2. The active material is precipitated at alcohol concentration of 80–85 per cent, pH 5.5 with acetic acid.

3. The precipitate is dried with absolute alcohol and ether. Yield 12–15 grams.

4. Fifty grams of the powder are extracted with three liters of water. pH 4.5

5. The active material is precipitated with acetone. The precipitate

is extracted with one liter of 1 per cent saline solution and the insoluble material discarded.

6. The solution is brought to 0.5 saturation with $(NH_4)_2SO_4$ by addition of 1 volume of saturated $(NH_4)_2SO_4$ solution. The precipitate is redissolved and reprecipitated twice. The precipitate contains LH and the supernatant FSH.

7. Precipitate is made up to 800 cc. with water. 100 cc. of saturated $(NH_4)_2SO_4$ are then added. The precipitate is discarded.

8. The supernatant is brought to 0.4 saturation.

9. Steps 7 and 8 are repeated twice. Final precipitation is made from 500 cc.

10. The precipitate from 9 is dissolved in 300 cc. of solution and brought to 0.37 salt saturation by addition of 175 cc. saturated ammonium sulfate. Precipitate removed.

11. Salt concentration increased to 0.4 saturation. Precipitate contains active material.

12. Steps 10 and 11 repeated twice from smaller volume.

13. Final product was dissolved in water to a 1 per cent solution and 10 per cent trichloroacetic acid added to 2.5 per cent.

14. Precipitate is dissolved in small volume of alkaline solution and reprecipitated as in 13.

15. Final precipitate is dissolved in alkali and dialyzed against distilled water.

16. Precipitate was obtained in dry form by evaporation of a frozen block.

This material contains 100 to 200 rat units per milligram, the unit being based on the repair of the interstitial tissue of the ovaries of hypophysectomized rats. The yield of active material is but from 4 to 8 per cent if based on the activity of the starting material. It is free of all other pituitary hormones such as lactogenic, adrenotropic, growth, thyrotropic and the follicle-stimulating factors.

When this material was investigated electrophoretically it was found that after 90 minutes only one boundary was present. The mobility was 6.36×10^{-5} in phosphate buffer, pH 7.53, ionic strength 0.05 at 1.5° C.

In another publication[1b] in which an alternate method of preparation is given, the sedimentation constant of the pure protein is given as 3.6 S in a 1 per cent NaCl solution. The isoelectric point is given as pH 4.6 and the molecular weight as 40,000. Solubility studies are also reported as an indication of the purity of the protein preparation.

1b Li, C. H. & Evans, H. M.   Jour. Am. Chem. Soc. in press.

In the Harvard laboratories we have also attempted to carry out the fractionation and purification of the proteins of the anterior pituitary glands. This joint research was undertaken because of the wide experience with the hormones of this gland by certain of our colleagues and because of the conviction of others that there was "no theoretical obstacle to the isolation of all the protein constituents of any given tissue. or to their characterization as chemical substances, and to the study of their interactions as biological components."[55] "Only reagents and conditions which are known to have little, if any, deleterious effect on most proteins have been employed. The pH has been retained between 5.4 and 8.0. Only two ions, ammonium and sulfate, in cold aqueous solution, have been added throughout the process"[49] in its early stages. In further preparations phosphate buffers have been employed to control the pH, which was never less than 5.2.

[49] Fevold, H. L., Lee, M., Hisaw, F. L. & Cohn, E. J. Endocrinology 26: 999. 1940.

DIAGRAM 1

FRESH FROZEN SHEEP PITUITARY TISSUE (1)

Extract with dilute NH₄OH at pH 8

Extract (2) — — — — — *Residue* Discarded

Add (NH₄)₂SO₄ to 0.25 M, adjust pH to 5.4 with N /10 H₂SO₄

Solution (3) Growth, thyrotropic, follicle-stimulating and luteinizing hormone — — — *Precipitate (3)* Adrenotropic hormone

Adjust to pH 6.5–7.0 with NH₄OH Add (NH₄)₂SO₄ by dialysis to 2.0 M

Solution Follicle-stimulating and 70 per cent thyrotropic hormone — — — *Precipitate* Growth, 30 per cent thyrotropic and luteinizing hormone

Dissolve in distilled water Dialyze salt free

Solution (4) 30 per cent thyrotropic and luteinizing hormone — — — *Precipitate* Growth hormone

Recognizing that generally, in studies of this kind, "the limiting factor was never the chemical study of the active principle, but the unsatisfactory nature of the methods for bio-assay," the direction and responsibility for all bio-assay in this problem was undertaken by the Harvard Biological Laboratories and for chemical fractionation and characterization by the Department of Physical Chemistry at the Harvard Medical School.

The first step in this investigation was the "Separation of Five Anterior Pituitary Hormones into Different Fractions by Isoelectric and Ammonium Sulfate Precipitation," reported in our previously published paper.[49] DIAGRAM 1 presents schematically the extraction, concentration and gross separation of the luteinizing hormone from the adrenotropic, growth, thyrotropic and follicle-stimulating hormone.

Traces of protein in (4) precipitable at pH 5.4 may conveniently be removed at this point, the reaction being immediately readjusted to pH 7. Thereafter, besides the luteinizing hormone, the main remaining hormone is roughly 30 per cent of the thyrotropic, which is in our crude solution (4). The thyrotropic hormone is more soluble in ammonium sulfate than is the luteinizing hormone. It can thus be removed by repetition of the precipitation with ammonium sulfate at the appropriate salt concentration yielding our final fraction (4).

TABLE 2 gives the solids and yields of various hormones in the first three steps of the procedure. Preparations III, IV, V and VII were prepared in the Harvard laboratories[50]. We are indebted to G. H. A. Clowes and J. C. Leighty for carrying out the extraction and initial fractionation of preparations XI, XII, XIII and XIV at the Eli Lilly and Company Laboratories.

Although the solids in solution (3) as prepared by J. C. Leighty were somewhat higher than in our smaller preparations, so, in many cases, were the activities, and it was a great satisfaction to discover that the process we had employed could readily be carried out by independent workers in another laboratory. The salting out of various hormones by ammonium sulfate has been carried out with great care by dialyzing the electrolyte through rotating collodion membranes at constant temperature and pH. By avoiding local excess of reagents by this procedure, denaturation of the various proteins is much diminished on the one hand; on the other, far cleaner separations between the proteins present in each

TABLE 2

Extraction of Anterior Pituitary Hormones*

Preparation	Solids[a] (gm/kg)	Adrenotropic hormone (RU/kg)	Growth hormone (RU/kg)	Luteinizing hormone (RU/kg)	Thyrotropic hormone (CU/kg)	Follicle-stimulating hormone[b] (RU/kg)
pH 8.0 Extract (2)						
III	59.8		50,000	8,000		(2,500)
IV	58.0		52,000	12,000	2,000	(3,000)
V	82.5	5,000	52,000	18,000	3,000	(2,000)
VII	71.2	5,000	50,000	20,000	4,000	(3,000)
pH 5.4 Precipitate (3) in 0.25 M (NH₄)₂SO₄						
III	40.4	5,000	none[c]	none[c]	none[c]	none[c]
IV	35.4	5,000	none	none	none	none
V	59.4	5,000	none	none	none	none
VII	48.1					none
pH 5.4 Solution (3) in 0.25 M (NH₄)₂SO₄						
III	19.4	none[c]	76,000	8,000		(2,500)
IV	22.6	none	112,000	14,000	2,000	(3,000)
V	22.0	none		16,000	3,000	(2,000)
VII	23.8			20,000	4,000	(3,000)
XI	32.9		74,000	26,000	4,000	(6,000)
XII	36.0		43,000	13,000	2,500	(6,000)
XIII	28.0[d]		36,000[d]	8,000[d]	4,500	(5,000)
XIV				48,000	4,000	

a All solids were determined by dry weight of aliquots after dialysis. Assay units are per kg. of fresh gland.
b Values given are only approximations, calculated from the number of units of follicle-stimulating hormone recovered after separation from luteinizing hormone. No method is available for the accurate determination of follicle-stimulating hormone in the presence of luteinizing hormone.
c The term "none" is applied when the response was unsatisfactory to maximum amounts of material reasonably tolerated by the test animal.
d One fraction of this preparation was lost in shipment.
e Bio-assays of the growth hormone were carried out by Milton Lee, who had collaborated throughout in this phase of our anterior pituitary hormone investigations. Bio-assay of the thyrotropic hormone was carried out by A. A. Abramowitz, and for the luteinizing and follicle-stimulating hormones by H. L. Fevold, both in collaboration with F. L. Hisaw in the Biological Laboratories.

system are effected at each ionic strength.[51][56-59] The results of this process as carried out in our earlier preparations have already been published.[49] Comparable studies with preparations XI, XII and XIII are reported in TABLE 3. Although there is considerable deviation in the results from preparation to preparation, this comparison of four different preparations reveals that the previously reported separations can readily be carried out on a far larger scale than we have heretofore reported.

By far the highest activity of the luteinizing hormone was found in the fractions precipitated by ammonium sulfate at 1.6 and 1.8M respectively. Indeed, in preparations XI, XII and XIII, less than 1,000 RU/kg. were found in the fractions precipitated beyond 2M ammonium sulfate. Moreover, the amounts of solids in these most active fractions were smaller than those from the soluble proteins precipitated by ammonium sulfate at either the lower ammonium sulfate concentration of 1.2 or at concentrations beyond 2.4. We therefore carried out the further purification of the luteinizing hormone by again precipitating the uncombined water-soluble proteins precipitated respectively at 1.2, 1.4, 1.6 and 1.8M ammonium sulfate. As an example of procedure we shall give in detail the purification of preparation XI derived from 6 kg. of frozen tissue. The total solids in this preparation at this point were 21.3 grams and the activity in RU/kg. was estimated to be 6.5, or within the levels that we have previously reported[49] for this crude luteinizing hormone fraction of from 5–10 RU mg. Several preparations have been more potent at this stage.

The above solution was again fractionated with ammonium sulfate at pH 6.8–7.0, precipitates removed respectively at ammonium sulfate concentration of 1.3, 1.95 and 2.6, the ammonium sulfate being added through a cellophane membrane as before. At 1.3M, 1.4 grams of protein precipitated. At 1.95, 11.5 grams precipitated and at 2.6, 6.4 grams. The first fraction contained less of the luteinizing hormone than we could test for; the last only traces. The fraction precipitating from 1.3–1.95 contained just over half of the protein, thus containing by far the greater part of the activity.

The middle fraction was again dissolved and precipitated with am-

[51] Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M. & Hughes, W. L. Jour. Am. Chem. Soc. 62: 3386. 1940.
[52] Evans, H. M., Simpson, M. E. & Pencharz, R. I. Cold Spring Harbor Symposia on Quant. Biol. 5: 229. 1937.
[49] Evans, H. M. Addresses at the Dedication of the Research Building of Abbott Laboratories, p. 20.
[54] Greep, R. O., van Dyke, H. B. & Chow, G. F. Am. Jour. Physiol. 133: 303. 1941.
[55] Cohn, E. J. Bull. N. Y. Acad. Med. 15: 639. 1939.
[56] Pillemer, L., Ecker, E. E., Oncley, J. L. & Cohn, E. J. Jour. Exp. Med. 74: 297. 1941.
[57] McMeekin, T. L. Jour. Am. Chem. Soc. 61: 2884. 1939.
[58] McMeekin, T. L. Jour. Am. Chem. Soc. 62: 3393. 1940.
[59] Cohn, E. J., Luetscher, J. A., Jr., Oncley, J. L., Armstrong, S. H., Jr. & Davis, B. D. Jour. Am. Chem. Soc. 62: 3396. 1940.

TABLE 3

FRACTIONATION OF pH 5.4, 0.25 M (NH₄)₂SO₄-SOLUBLE PITUITARY EXTRACTIVES WITH (NH₄)₂SO₄ AT pH 6.5–7.0

Concentration of $NH_4)_3SO_4$	Globulins precipitated	Growth hormone	Soluble proteins	Luteinizing hormone	Thyrotropin	Follicle-stimulating hormone
mols L	*gm kg*	*RU kg*	*gm kg*[a]	*RU kg*	*CU kg*	*RU kg*
		(See Table 2 and Ref. 49)				
1.4	6.13	25,000	0.51	2,000	none	none
1.6	1.66	64,000	0.31	4,000	none	none
1.8	1.21	trace	0.63	4,000	300	none
2.0	0.64	none	0.61	4,000	350	none
2.2	0.34	none	0.49	1,000	1,500	none
2.4	0.32	none	0.66	500	trace	trace
2.6	0.28	none	0.81	trace	none	1,000
2.8	0.40	none	0.75	none	none	2,000
3.8	1.88	none	2.33	none	none	none
		Preparation XI				
1.2	11.35	11,000	1.05	3,000	200	none
1.4	4.80	25,000	0.60	10,000	100	none
1.6	2.40	26,000	1.00	4,000	300	none
1.8	1.26	2,000	0.90	6,000	550	none
2.4	2.05	5,000	3.11	—	2,500	traces
3.0	2.21	none	2.14	—	100	1,000
		Preparation XII				
1.2	12.05	—	0.75	1,000	none	—
1.4	4.50	76,000	0.35	4,000	none	—
1.6	3.84	6,000	0.46	4,000	500	—
1.8	3.34	3,800	0.62	6,000	600	—
2.4	2.35	2,800	—	—	1,300	2,000
3.0	2.31	—	—	—	—	4,000
		Preparation XIII				
1.2	9.28	4,400	0.80	800	none	—
1.4	*	*	*	—	—	—
1.6	2.96	6,300	0.94	2,000	800	—
1.8	2.96	2,400	0.74	4,000	800	traces
2.4	2.24	—	3.80	1,000	2,800	2,000
3.0	2.30	—	—	—	none	4,000

[a] Dry weight of aliquots after dialysis. Assay units are given per kilogram of fresh gland.
* Lost in shipment.

monium sulfate from a still smaller volume. Since the total amount of protein in this system was smaller, the volume was also maintained smaller and fractions removed respectively at 1.4, 1.7 and 2.6 M. The first and the last contained only traces of the luteinizing hormone, which was thus again largely concentrated in the middle fraction which contained 3.9 grams. Since the total solids were reduced from 21.3 to 3.9 grams, an increase in activity from 6.5 to 35.5 RU mg. would result were the yields of luteinizing hormone quantitative. Actually, the losses in

carrying out this process have always been small, yielding fractions with activities varying from 30 to 40 RU mg. as previously reported.[49]

Further concentration of the luteinizing hormone has been accomplished by dissolving the material with activities of 30–40 RU 'mg. in water, dialyzing salt free and carefully adjusting to pH 5.0–5.2 with dilute H_2SO_4. A precipitate with comparatively small activity (about 10 per cent of the total) was removed. The supernatant solution was precipitated with $(NH_4)_2SO_4$ at pH 7 in order to concentrate the protein, redissolved in about 17 cc. of water per kilogram of fresh tissue, and again dialyzed salt free and adjusted to pH 5.0–5.2, when a further precipitate was removed. This entire process was then repeated a third time. Approximately one quarter of the original protein remained in solution at pH 5.2 after this third precipitation from small volume. Were the yield of hormone quantitative. an activity of from 120–160 RU mg. would be achieved. Bio-assay revealed losses of from 10 to 20 per cent; the activity achieved should thus have been of the order of 100–140 RU 'mg. Actually, bio-assays have revealed even greater activity at this stage, and the solids from 6 kilograms of fresh frozen sheep pituitary glands have been reduced to less than one gram.

This material was studied electrophoretically in a phosphate buffer of pH 7.7 and ionic strength 0.2. The schlieren diagram obtained reveals two and perhaps three components with mobilities of approximately 2.0, 4.0 and 5.4 $\times$ 10^{-5} cm²/volt sec.

This concentrated solution has been the starting material in our further efforts to purify the luteinizing hormone. It was next dialyzed against phosphate buffer of pH 5.6 and 0.25 ionic strength until equilibrium was reached, and then dialyzed against distilled water and finally conductivity water. The pH was then 5.9–6.0. Under these conditions a euglobulin separated from the concentrated solution. The precipitated globulin was removed. and the supernatant solution concentrated by negative pressure dialysis to a small volume (15 cc. per kg. of frozen tissue). This was further chilled to 0° C. and the precipitated globulin combined with the main globulin precipitate.

The precipitated globulin. in a volume of 1 cc. kg. of frozen tissue, was extracted with a small quantity of 1.18M ammonium sulfate. at pH 6 and 0° C. This solution was separated from the undissolved precipitate by centrifugation at 0° C. and then brought to 25° C. when a precipitate containing most of the luteinizing hormone separated. The supernatant was next cooled to 0° C. and employed in a second extract of the original precipitate again at 0° C. This process was repeated as often as any material was precipitated at 25° C. under these conditions. The pre-

cipitates obtained by the increase in temperature were collected and contained luteinizing hormone.

Active material was thus obtained as, or associated with, a globulin with a negative temperature coefficient between 0° and 25° C. at pH 6 in 1.16M ammonium sulfate. The amount of material obtained at this point was roughly of the order of 10 mg., kg. of fresh pituitary tissue. The fractions with activity above 150 RU/mg. have not been prepared sufficiently often for us to report values for the activity of this globulin at this time. Although great significance cannot be attached to the physical constants of this fraction since it has not been prepared often enough, in the interests of other workers in the field it may be helpful to report the ultracentrifugal and electrophoretic measurements that have been made.

Examination in the ultracentrifuge carried out by J. L. Oncley over a considerable range of protein concentration showed that the protein in this globulin fraction was not homogeneous with respect to size and shape. Components of different sedimentation constant were present in a 1.3 per cent solution in 0.2M KCl. A slower moving component comprised approximately 60 per cent of the total and had a sedimentation constant of about 3.3 S, and the remainder of the material had a sedimentation constant of about 6.6 S. A preliminary study in a divided ultracentrifuge cell indicated that the fraction of lower sedimentation constant was active. Whether the fraction of higher sedimentation constant was active has not been established.

Electrophoretic analysis of this globulin fraction at a protein concentration of 0.4 per cent in phosphate buffer of pH 7.7 and ionic strength 0.2 also revealed at least two components. Of these, the slower moving, estimated to be about 75 per cent of the total, had an electrophoretic mobility of about 1.8×10^{-5}, while the faster moving component, comprising approximately 25 per cent of the total, had a mobility of about 5.3×10^{-5}. Electrophoretic separation of the two components, carried out by S. H. Armstrong, Jr., suggested that the fast moving material was relatively inactive while the slow moving fraction appeared the more active.

These observations thus suggested that the highest activity we have noted was in the component of smaller sedimentation constant and lower electrophoretic mobility. It is always possible, however, that none of the protein components revealed by the schlieren diagrams represent the active hormone.

This work was interrupted at this point by my acceptance of a govern-

ment post and repetition of the experiments at Harvard has been postponed because of preoccupation with protein problems related to defense.

COMPARISON OF LUTEINIZING HORMONE PREPARATION

A comparison of the physical constants of the luteinizing hormone preparations from the three laboratories is presented in TABLE 5. It is apparent that if we assume that the proteins isolated and studied by the Squibb and Rockefeller Institute group, and by the University of California group, are pure luteinizing hormone proteins, we must come to the conclusion that sheep and swine luteinizing hormones are two different proteins. Chemical analysis of the two isolated proteins from sheep and swine pituitaries also brings out the fact that they are different proteins. The sheep protein isolated by the University of California group contains 4.5 per cent mannose, 5.8 per cent glucosamine, 1 per cent tryptophane, 4.5 per cent tyrosine, and 5.4 per cent cystine. The protein isolated by the Squibb and Rockefeller Institute group from swine pituitary contains only 2 per cent carbohydrate, but 3.8 per cent tryptophane. The other constituents mentioned above have not been determined for swine preparations.

TABLE 5
COMPARISON OF LUTEINIZING HORMONE PREPARATIONS

	Iso-electric point pH	Electro-phoretic mobility 0° C., $\times 10^5$	Sedimentation constant. Svedberg Units, S	Molecular weight
Squibb and Rockefeller Institute (fresh swine pituitary)	7.4	0.6[a]	5.4[d]	90,000[f]
University of California (acetone-dried sheep pituitary)	4.6	6.4[b]	3.6[d]	40,000[g]
Harvard University (fresh sheep pituitary)	—	1.8–2.0[c]	3.3; 6.6[e]	

[a] In 0.005 ionic strength, diethylbarbiturate buffer, pH 7.86.
[b] In 0.05 ionic strength, phosphate buffer, pH 7.53.
[c] In 0.02 ionic strength, phosphate buffer, pH 7.77.
[d] In 1 per cent (0.17M) NaCl.
[e] In 0.20M KCl.
[f] From sedimentation velocity and diffusion measurement.
[g] From osmotic pressure measurements.

If we consider the work on sheep pituitary tissue, the agreement is also not too satisfactory. The sedimentation constant of our slow sedimenting component is close to that reported by the University of California workers. The electrophoretic mobilities reported by the two

laboratories do not agree, and the difference would appear greater than that expected from the difference in ionic strength and protein concentration used in the separate investigations. The higher mobility observed by the University of California workers is of the same magnitude as the faster moving electrophoretic component of relatively low activity observed in our work, however.

The activity data are even more difficult to reconcile, and would appear to be greater than can be explained by the different methods of bio-assay employed in different laboratories.

NOMENCLATURE OF THE LUTEINIZING HORMONE

In conclusion, a few words should be said regarding the nomenclature of the luteinizing hormone. The pituitary gonadotropic hormones were originally given names which defined their physiological actions in the female, namely follicle-stimulating and luteinizing hormones (FSH and LH).[5-7] It was subsequently shown that these two factors stimulated spermatogenesis and interstitial-cell hypertrophy respectively in the male.[10][11]

Evans and coworkers reported that the factor which stimulates the interstitial cells was not the factor which produces luteinization. They named this new substance the interstitial-cell-stimulating hormone (ICSH) and reported a complete separation of ICSH and LH.[9,52] This claim was later withdrawn[53] but the term ICSH has been retained by them for the substance which produces both luteinization and interstitial-cell stimulation, in other words for the luteinizing hormone. The terms LH and ICSH are now synonymous and refer to the same substance.

The Squibb group has recently preferred to devise Greek names for the two substances and have called them "thylakentrin" and "metakentrin"; thylakentrin referring to the FSH and metakentrin to the LH.[51]

The luteinizing hormone is therefore identified with three names in the literature: LH, which was the name originally given to the hormone, ICSH, and metakentrin.

THE LACTOGENIC HORMONE AND MAMMOGEN*

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Section I: The Lactogenic Hormone

INTRODUCTION

The first experimental evidence for the presence of a hormone or hormones in the anterior pituitary body which are essential for the initiation and maintenance of lactation was presented by Stricker and Grüter[1] in 1928. The existence of a specific, anterior lobe, lactogenic hormone is generally accepted at the present time. Various names have been proposed for this hormone: prolactin (Riddle), galactin (Turner), and mammotropin (Lyons). It appears that prolactin has been most widely used. It is not intended in the present discussion to consider whether or not prolactin is *the* lactogenic hormone; the term prolactin will be employed to designate *a* lactogenic hormone of the anterior pituitary. The ability of this substance to initiate, in a variety of species, lactation in glands presumably possessing a certain amount of alveolar development has been established by numerous investigators.[2-4]

The fact that prolactin was the first anterior pituitary hormone to be obtained in a highly purified state and as an apparently homogeneous preparation resulted in a considerable accumulation of physical-chemical and chemical data for this hormone. In order to permit adequate discussion of this material, the physiological aspects of prolactin will not be detailed. It may be added that the physiologists have far outstripped the chemists in the rate of production of new facts regarding the anterior pituitary hormones. Consequently, there have been exhaustive treatments of the physiology of prolactin,[2-5] and repetition of this material at the present time would contribute little to this symposium. The methods of assay and preparation of prolactin will be only mentioned, and detailed descriptions of experimental procedures by which other data were obtained will be left for presentation elsewhere.

[1] Stricker, P., & Grüter, F. Comp. rend. Soc. Biol. 99: 1978. 1928.
[2] Nelson, W. O. Physiol. Rev. 16: 488. 1936.
[3] Turner, C. W. In "Sex and Internal Secretions," 2nd edit., E. Allen, Ed. Williams and Wilkins Co. Baltimore. 1939. p. 740.
[4] Folley, S. J. Biol. Rev. 16: 421. 1940.
[5] Riddle, O., & Bates, R. W. In "Sex and Internal Secretions," 2nd. edit., E. Allen, Ed. Williams and Wilkins Co. Baltimore. 1939. p. 1088.

It is desired to acknowledge gratefully the stimulating collaboration and support given this work by Professor C. N. H. Long.   Dr. R. W. Bonsnes contributed to certain of the data obtained for purified prolactin, which are to be discussed.   Dr. H. R. Catchpole conducted some of the early bio-assays and Miss Hilda B. Ritter subsequently collaborated in the many bio-assays which have been done in the course of this work. Studies on the concentration and isolation from natural sources of substances which may occur in relatively small amounts are greatly facilitated by the cooperative efforts of a number of individuals.   This is particularly emphasized when progress must be evaluated by biological assays which are often laborious.

METHODS OF ASSAY OF PROLACTIN

The methods generally employed for the assay of prolactin may be classified into two groups: (1) crop-sac or crop-gland methods, and (2) lactation or mammary gland methods.   These methods have been discussed in detail by Bates,[6] Riddle and Bates,[5] and Lyons.[7a]   The two laboratories represented by these workers, and that of the National Institute for Research in Dairying at Reading, England, as exemplified in the investigations of Folley and coworkers,[8] have examined in great detail factors influencing the assay of prolactin.   These descriptions, together with the availability of an International Standard of an accepted unitage makes possible the highly desirable comparison of products obtained by various investigators.

Prolactin activities in the work to be described have been determined by two methods: (a) the 2-day "local" assay, and (b) the 4-day systemic test.[7]   It has been observed that the systemic method has given more reproducible and consistent results, and this technique has therefore been generally employed.   Six-weeks-old pigeons of a single strain (White Carneau) and from a single source (Palmetto Pigeon Plant, Sumter, South Carolina) have been used.   Comparisons have been made with the International Standard, kindly supplied by Dr. A. S. Parkes, with an accepted activity of 10 International Units per mg.   It may be mentioned that, in agreement with the recent publication of Lyons,[7b] it has been found that the activity claimed for the International Standard is approximately three times too high.   Our preparations were initially evaluated in terms of absolute activity and the impression therefore arose that certain amorphous prolactin preparations were approximately

⁵ **Bates, R. W.**   Cold Spring Harbor Symposia on Quantitative Biology **5**: 91.   1937.
⁷ᵃ **Lyons, W. R.**   Cold Spring Harbor Symposia on Quantitative Biology **5**: 198.   1937.
⁷ᵇ **Lyons, W. R.**   Endocrinology **28**: 161.   1941.
⁸ **Folley, S. J., Dyer, F. J., & Coward, K. H.**   Jour. Endocrinology **2**: 179.   1940.

two to three times as active as the products we obtained. It is of course necessary and desirable, for purposes of comparison with other investigators, to accept the value of 10 International Units per mg. for the International Standard in evaluating the activity of unknown products. This has been done in obtaining bio-assay data presented here.

PREPARATION OF PROLACTIN

Extracts highly active in prolactin may be prepared from either fresh or acetone-desiccated pituitary glands by acid or alkaline extraction, with aqueous or aqueous-organic solvents. Bergman and Turner[9] compared 4 frequently employed methods of extraction of the lactogenic hormone and found the alkaline 60 to 70 per cent ethanol extraction procedure to be superior for both total yield and unitage per mg. of extracted solids. Though it is true that an alkaline extraction procedure is efficient in removing a very considerable proportion of the prolactin present in pituitary tissue, alkaline solvents also effect solution of relatively large amounts of extraneous tissue proteins. For this reason, the acid-acetone technique of Lyons[10] has been employed in the investigations in our laboratory. This excellent method is relatively simple and gives a product from which a highly purified preparation of prolactin may be obtained. The chief attribute of Lyons' method is the fact that the crude prolactin obtained is quite free from other pituitary proteins, the chief contaminant being one other pituitary hormone of considerable interest, namely, the adrenotropic factor.

One kg. of whole beef pituitary glands is extracted as described by Lyons.[7a,10] The acetone concentration of the acid-acetone extract obtained is increased to 92 per cent and the mixture allowed to stand overnight in the icebox. The precipitate is separated by centrifuging. Some purification is effected by extraction of the precipitate with 4-50 ml. portions of water, separating the extract each time from a water-insoluble residue by centrifuging, combining the clear extracts and adding acetone to a concentration of 92 per cent. After being chilled overnight the precipitate is separated by centrifugation, washed thoroughly with acetone and dried *in vacuo* over sulfuric acid at room temperature. Range of yields from 1 kg. of glands is 1.2 to 1.9 gm.

Purification of Prolactin

The product obtained as described contains a considerable quantity of protein which exhibits a minimal solubility at pH 6.4 to 6.8. This is

[9] Bergman, A. J., & Turner, C. W. Jour. Biol. Chem. 115: 247. 1937.
[10] Lyons, W. R. Proc. Soc. Exp. Biol. Med. 35: 645. 1936–37.

the so-called adrenotropic fraction.[10]   A complete separation of the latter material from prolactin may be effected by solution of the crude product at approximately pH 8.0, with the aid of 0.1 N sodium hydroxide, adjusting to pH 6.6 by the addition of 0.1 N hydrochloric acid, and removing the precipitate which forms.   Prolactin is obtained from the supernatant by lowering the pH to 5.4 by further addition of the dilute acid.   The precipitate of prolactin is then redissolved in dilute alkaline solution as before and any precipitate which separates above pH 6.0 is removed.   By repeating this procedure several times, and gradually reducing the volumes of the solutions employed, a very effective purification of prolactin results.   Better separations of adrentropic protein and prolactin, and somewhat larger yields of the latter have been obtained by the use of ammonium sulfate together with adjustment of hydrogen ion concentration.   Both the adrenotropic and prolactin fractions have a significant degree of solubility at the hydrogen ion concentrations at which they exhibit maximum flocculation.   The presence of salt diminishes loss of prolactin in supernatants, and at the same time increases the completeness of separation of the adrenotropic fraction.   A flow sheet indicating the preparative procedure employed is shown in CHART 1.

CRYSTALLIZATION OF PROLACTIN

Several years ago a preliminary communication from this laboratory[11] reported that a crystalline protein had been obtained from a highly purified prolactin product prepared by a procedure essentially that described by Lyons.[7,1]   The yield of crystalline material was small, due chiefly to denaturation of the protein which occurred during the crystallization procedure.   This fact, together with subsequent observations that the original procedure described[11] has not been uniformly successful, has rather diminished emphasis on crystallization of the protein.   This has been particularly true in view of the fact that, in the case of proteins, the crystalline state is not an adequate criterion of purity.   Indeed, recrystallization of prolactin has frequently been accompanied by a decrease in biological potency and a diminished solubility of the protein.   Similar observations have been made independently in the Schering Laboratories by Dr. Erwin Schwenk and Dr. Gerhard Fleischer, who have informed the writer that they have been successful in obtaining crystalline preparations of prolactin.

Two methods have been employed for obtaining crystalline products,

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[11] White, A., Catchpole, H. R., & Long, C. N. H. Science 86: 82. 1937.

PREPARATION OF PROLACTIN

I KG. BEEF PITUITARY GLANDS

Step 1. → Acid-acetone extraction (pH 1.5

residue   supernatant
discarded)   +
                         Step 2 → Acetone to 92% concentration

Step 3 → residue   supernatant
          Extraction with water   (discarded)

residue   solution
discarded)   +
                 Step 4 → Acetone to 92% concentration

supernatant   precipitate
(discarded)   Crude prolactin: yield. 1.2 to 1.9 grams
                                               +
                    Step 5 → Solution in aqueous alkali
                             adjust to pH 6.6 in presence
                             of .05 saturation with (NH4)2SO4

superratant   precipitate
                                           (adrenotropic)
Step 6 → Adjust to pH 5.4

Steps 5 and 6 repeated several times.
Final pH 5.4 precipitate;
    dialyze free of salt - dry with acetone or lyophilize.
Yield purified prolactin: 120 to 190 mgs.

CHART 1. Preparation of Prolactin.

viz., (a) an acetic acid-pyridine procedure which is essentially that described[12] for the crystallization of insulin, and (b) precipitation from dilute acetone solutions. Some variation in concentrations or acetic acid and pyridine, as compared to those prescribed for the insulin crystallization, has been used because clearer supernatants, from which crystals deposit, are obtained. Dr. Schwenk states that the crystallization method employed in the Schering Laboratories is essentially the tech-

12 du Vigneaud, V., Jensen, H., & Wintersteiner, O. Jour. Pharm. Exp. Ther. **32**: 367. 1927–28.
13 Abel, J. J., Geiling, E. M. K., Rouiller, C. A., Bell, F. K., & Wintersteiner, O. Jour. Pharm. Exp. Ther **81**: 65. 1927.

nique published by Abel and his colleagues,[1v] with the modification of omitting brucine from the procedure.

A few remarks may be made regarding the crystallization procedures:

1) Either technique yields crystalline material which is essentially the same in microscopic appearance. The latter is most frequently that of small hexagonal crystals. Clearer definition of crystalline form has been obtained after longer periods of standing at low temperatures, although this observation is not a consistent one. The preparations under the microscope frequently have the appearance of prismatic crystals, the edges of which are somewhat rounded. The crystals tend to dissolve slowly as the solution warms to room temperature. A photomicrograph of a preparation which had been permitted to deposit over a 2-month period at low temperature is shown in FIGURE 1.

(2) The yield of crystalline product is exceedingly small. In the case of the pyridine-acetic acid procedure the small yield may be attributed largely to the fact that purified prolactin appears rapidly to lose its solubility when subjected to repeated re-solutions in dilute acetic acid; this is necessary to get some of the protein into the pyridine-acetic acid mixture. The insoluble residue remaining after 10 reprecipitations of the protein, as is done in the crystallization method, amounts to 50 to 65 per cent of amorphous prolactin taken. Bio-assay of this insoluble residue indicates that a loss of 50 to 80 per cent of the physiological potency generally

FIGURE 1.   Crystalline prolactin.   X 900 diam.

accompanies loss in solubility of prolactin occurring in this step of the crystallization procedure.   The limited solubility of the prolactin under the conditions employed has contributed to the low yield of crystalline protein.

3) Recrystallization has not been satisfactory because of apparent denaturation of the protein, associated frequently with a decrease in biological potency.   Recrystallizations which have been successful have been carried out by the pyridine-acetic acid procedure.

(4) The dilute acetone technique suffers also from the drawback of low yield of crystalline product.   The procedure takes advantage of the fact that prolactin exhibits a considerable degree of solubility when precipitated from slightly alkaline solution by adjustment of the hydrogen ion concentration to the point of maximum flocculation.   The protein remaining in solution may be precipitated from the supernatant fluid by the addition of acetone to a concentration of 80 per cent, followed by chilling of the solution.

The prolactin activities of the various products are presented in TABLE 1.   For purposes of comparison, two prolactin preparations from other laboratories have been carefully assayed.   Each of these was prepared from sheep pituitary glands; one was kindly furnished by Dr. Schwenk and the other was obtained through the generosity of Dr. W. R. Lyons of the University of California.   It will be seen from the data in TABLE 1 that several preparations of purified prolactin, prepared in three different laboratories, have a biological activity which, within the limits of error of the assay method, may be considered identical.

TABLE 1

PROLACTIN ACTIVITY OF VARIOUS PREPARATIONS

Preparation	International Units per mg.
Crude prolactin	10–15
Purified prolactin	30–35
Crystalline prolactin (pyridine-acetic acid procedure)	30–35
Crystalline prolactin (acetone procedure)	30
Twice recrystallized prolactin (pyridine-acetic acid procedure)	30–35
Purified prolactin*	30–40
Purified prolactin**	30

* Obtained from Dr. Schwenk.
** Obtained from Dr. Lyons.

HOMOGENEITY STUDIES

The methods generally accepted as useful for examining the homogeneity of proteins are (a) the Tiselius electrophoresis technique, (b) solubility studies, and (c) ultracentrifugal analysis. Each of these methods has been employed in the study of prolactin. All preparations have been obtained from beef pituitary glands.

Electrophoresis in the Tiselius Apparatus

The electrophoretic behavior of crystalline prolactin has been previously reported from this laboratory.[14] Since the initial publication, the apparatus has been equipped with the schlieren scanning device of Longsworth.[15] FIGURE 2 shows scanning patterns obtained in a typical experiment with a crystalline prolactin preparation (35 I.U./mg.). The protein boundary migrated in a manner characteristic of a homogeneous protein. Similar homogeneity was observed in an electrophoresis experiment conducted at pH 3.90 (acetate buffer; ionic strength 0.05).
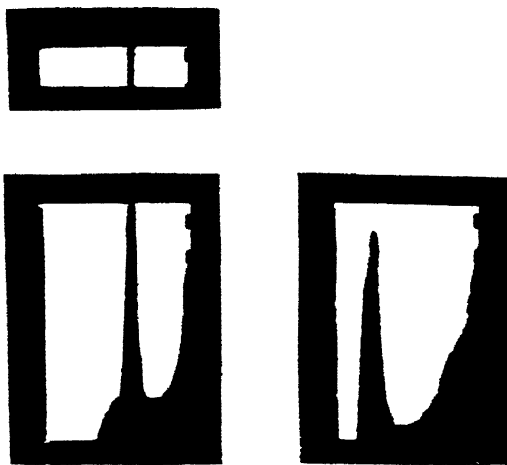

FIGURE 2. Electrophoretic patterns of the descending boundary in an experiment with crystalline prolactin. Protein concentration, 2 per cent; buffer, 0.017 M phosphate (pH 8.0); ionic strength, 0.1; temperature, 6° C. Photographs, from left to right, at zero time and after 4 hours.

The homogeneity and mobility of highly purified prolactin preparations from both beef and sheep pituitary glands have been studied in some detail by Li, Lyons, and Evans.[16] These investigators have demon-

[14] Shipley, R. A., Stern, K. G., & White, A. Jour. Exp. Med. 69: 785. 1939.
[15] Longsworth, L. G. Jour. Am. Chem. Soc. 61: 529. 1939.
[16a] Li, C. H., Lyons, W. R., & Evans, H. M. Jour. Gen. Physiol. 23: 433. 1940.
[16b] Jour. Am. Chem. Soc. 62: 2925. 1940.
[16c] Jour. Biol. Chem. 140: 43. 1941.

strated that the lactogenic products prepared from these two different species are homogeneous and indistinguishable in their electrophoretic behavior.

Solubility Studies

Solubility studies supporting the conclusion that their highly purified, amorphous prolactin is homogeneous have been published by Li, Lyons, and Evans[17] who reported that although both sheep and beef prolactin preparations behaved as pure substances, they exhibited differences in solubility, thus indicating a species specificity. Crystalline prolactin with a physiological activity of 30 to 35 International Units per mg. has been studied in 3 series of solubility experiments, each with a different solvent: (a) 0.12M NaCl in 0.01N HCl, (b) 0.33M NaCl in 0.01N HCl, and (c) redistilled water. The pH values of these solutions were determined at 23° C. with the glass electrode and found to be 2.05, 2.0, and 6.92, respectively.

Approximately 500 mg. of protein were employed for each solubility study. The protein was precipitated at pH 5.5 and washed with successive portions of each solvent until 2 successive solubilities were the same. The precipitate was then broken into a fine suspension and varying quantities distributed among tubes filled with the solvent. The tubes were rotated for 48 hours at 23° C., filtered and the filtrate analyzed for nitrogen by the Kjeldahl micro-procedure. The results of the solubility studies are shown in FIGURE 3. It will be seen that the solubility is independent of the amount of the saturating solid, from the first appearance of a solid phase. No solid phase appeared until the break in each of the curves in FIGURE 3, *i.e.*, before the slope became zero. Bioassays of the soluble and insoluble material did not show any physiological differences.

Sedimentation

A 2 per cent solution of crystalline prolactin at pH 8.0 was made by dissolving the protein with the aid of 0.1N sodium hydroxide and adjusting with 0.1N hydrochloric acid. This solution was examined in an analytical air-driven ultracentrifuge[1b] arranged for optical study in the visible region by the Toepler schlieren method, as developed for the ultracentrifuge by Philpot.[19] The initial protein peak present at the start of the experiment sedimented at a uniform rate. A comparable run with highly purified, amorphous prolactin gave a similar diagram. In FIGURE 4 are reproduced the photographs obtained in a run with a crys-

[17] Li, C. H., Lyons, W. R., & Evans, H. M.   Jour. Gen. Physiol. **24**: 303.   1941.
[1b] Beams, J. W., Linke, F. W., & Sommer, P.   Rev. Sci. Instr. **9**: 246.   1938.
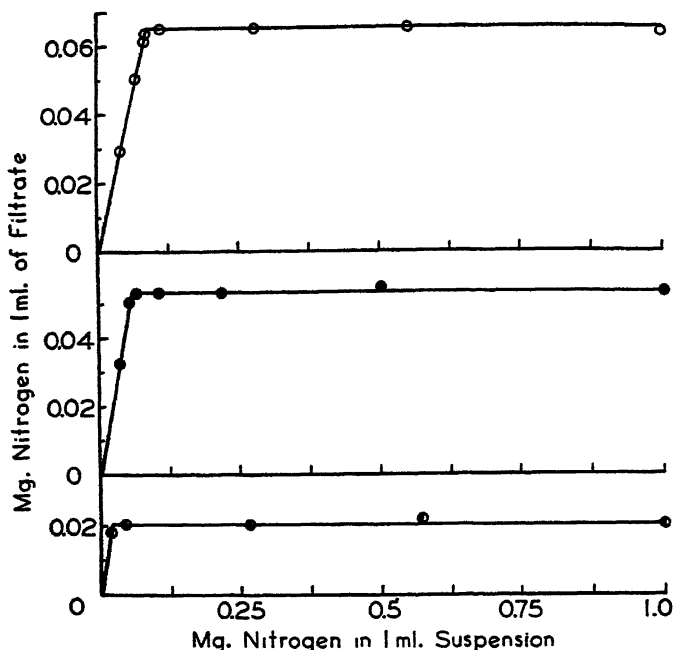[19] Philpot, J. S. L.   Nature **141**: 283.   1938.

talline preparation.   The average value of the sedimentation constant, s_{20}, found for prolaction in several runs was approximately 2.8 S.   If the prolactin molecule is tentatively assumed to be spherical, the sedimentation constant obtained indicates an approximate molecular weight[20] of the order of 35,000 for this protein hormone.

A highly purified, amorphous prolactin preparation (35 I.U./mg.) was sent to the laboratory of Professor J. W. Williams at the University of Wisconsin for examination in the Svedberg oil-driven ultracentrifuge. Several sedimentation and diffusion experiments have been completed.

[20] Svedberg, T., & Pedersen, K. O. "The Ultracentrifuge." Clarendon Press. Oxford. 1940. p. 406.

Professor Williams has written the following in a personal communication, "We have been able to finish a few sedimentation and diffusion experiments with your prolactin. The results we find are: $s_{20} = 2.65 s$, $D_{20} = 7.5 \times 10^{-7}$, and $M = 32,000$. I am inclined to think that the molecular weight value will eventually turn out to be a little larger than the figure we give. On sedimentation and diffusion the prolactin gave curves very much like those that one would expect when working with a simple homogeneous substance."

Li, Lyons, and Evans[16c] have recently determined the molecular weight of prolactin by osmotic pressure measurements and have also calculated the probable molecular weight on the basis of the cystine, arginine, tyrosine, tryptophane, and sulfur contents of the hormone. From these data the molecular weight of the lactogenic hormone was estimated to be approximately 25,000. It would appear evident on the basis of other information now available that this value is considerably too low.

The preliminary data obtained by Professor Williams and his colleagues will be extended when circumstances permit resumption of this work. It seems advisable to obtain as accurate physical-chemical constants as possible for any highly purified protein, and the physiological interest in prolactin adds importance to data obtained in a laboratory experienced in determining these constants. Grateful acknowledgment is made to Professor Williams and his colleagues for these preliminary figures.

ULTRAVIOLET ABSORPTION SPECTRUM

The ultraviolet absorption spectrum of prolactin has been measured by Dr. G. I. Lavin, of The Rockefeller Institute for Medical Research.[21] In FIGURE 5 are reproduced the absorption spectrum curves of crystalline prolactin and of a highly purified amorphous preparation. All the solutions were photographed at room temperature in aqueous medium at pH 7.4. The absorption curves were measured with the aid of a Spekker spectrophotometer and a small Hilger quartz spectrograph, with a tungsten steel spark as the light source.

The absorption curves obtained are typical of those found for a number of proteins, particularly those from animal tissue. The broad band with the maximum at about 2800 Å is to be attributed to the combined absorption of the amino acids, tryptophane, tyrosine, and phenylalanine, all of which have been demonstrated to be constituents of prolactin.

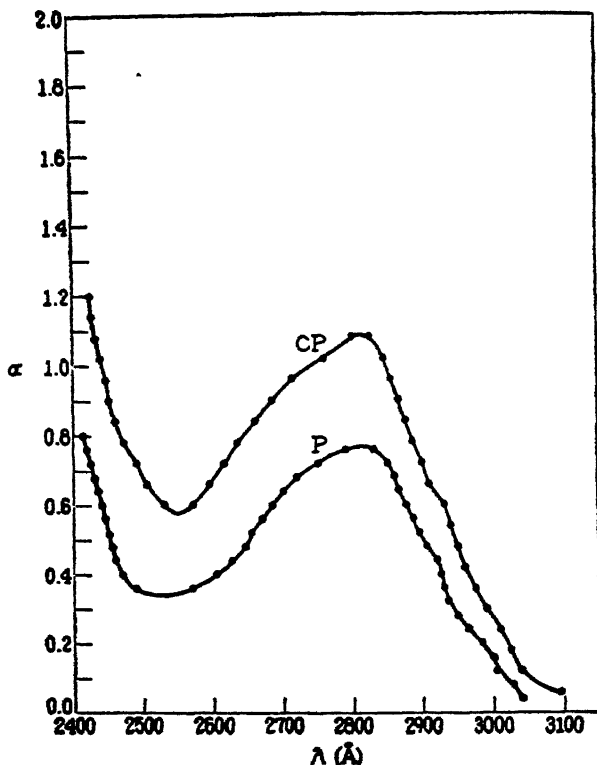[21] **White, A., & Lavin, G. I.** Jour. Biol. Chem. **132:** 717, 1940.

Figure 5. Absorption curves of solutions of prolactin. Curve C P represents crystalline prolactin; curve P was obtained with highly purified amorphous prolactin. pH 7.4. α (extinction coefficient α = 1 $d \log I_0'I$, c = concentration (2.5 mg. per cc. in the case of curve P; 1.8 mg. per cc. in the case of curve C P, l = cell thickness in cm. '1 cm.), I_o = initial intensity of light, I = intensity after passing through thickness l.

ISOELECTRIC POINT OF PROLACTIN

The isoelectric point of prolactin has been determined in two laboratories from mobility studies in the Tiselius apparatus. Values of pH 5.6,[14] pH 5.70,[16a] and pH 5.73[16b] have been reported. In view of the fact that the published values for the isoelectric point of prolactin have been based upon mobility studies in the Tiselius apparatus, it has seemed of interest to conduct a determination of the isoelectric point by the technique developed by Abramson and his colleagues.[22] This method is also an electrophoretic one, based upon direct measurement of the electrical mobility of microscopically visible quartz particles coated with an adsorbed layer of protein. The data obtained are plotted in FIGURE 6. It

22 Abramson, H. A. "Electrokinetic Phenomena in Biology and Medicine." Chemical Catalog Co., Inc. New York. 1934.
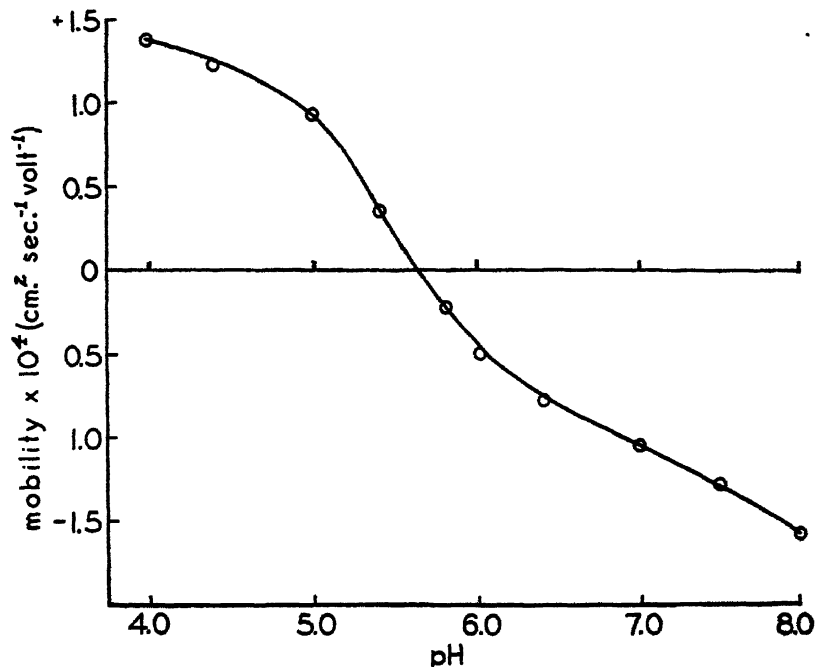
FIGURE 6. The electric mobility of quartz particles covered with prolactin in acetate and phosphate buffers of constant ionic strength (0.1). The isoelectric point is between pH 5.65 and pH 5.70.

will be seen that the isoelectric point of prolactin appears to be at pH 5.65, a value which is in good agreement with the previously published isoelectric point values obtained in the Tiselius apparatus.

ELEMENTARY ANALYSIS AND QUALITATIVE REACTIONS

Several preparations of crystalline and of highly purified, amorphous prolactin have been subjected to elemental microanalysis. These analyses have been conducted at intervals over the course of several years by Mr. J. F. Alicino on different preparations in order to check the constancy of composition and of analytical technique. The nitrogen values obtained by Mr. Alicino by means of the Dumas method have in all cases been checked in our laboratory by the Kjeldahl micro-procedure. In addition, a preparation of highly purified, amorphous prolactin obtained from Dr. Schwenk has also been quantitatively examined for its elemental composition. The data obtained are presented in TABLE 2, together with data obtained by Dr. Lyons and kindly provided by the latter investigator in a private communication. It will be seen that the elementary

composition of the prolactin preparations differs particularly in respect to its nitrogen content from the data reported several years ago.[11] The rather good analytical agreement among various preparations, taken together with the results of the bio-assays and certain unpublished physical-chemical and physiological results indicate that the prolactin products prepared in three different laboratories are probably the same protein.

TABLE 2
ELEMENTARY COMPOSITION OF VARIOUS PROLACTIN PREPARATIONS

Preparation	C	H	N	S	Ash
	per cent	per cent	per cent	per cent	per cent
Purified prolactin*	52.04	7.01	16.84	2.05	0.59
Crystalline prolactin*	51.81	6.81	16.49	2.03	0.50
Prolactin (Schwenk)*	51.10	7.00	16.61	1.98	0.72
Prolactin Lyons**	51.40	7.01	16.09	2.24	negligible
White, Catchpole and Long[11]*	51.11	6.76	14.38	1.77	"
Li, Lyons and Evans[16c]				1.79	

* Each of the analytical values for these preparations represents the average of two determinations. All values in the table are calculated on an ash- and moisture-free basis.
** Personal communication from Dr. Lyons.

The crystalline and the purified products give the usual protein color tests (biuret, xanthoproteic, Millon's, Hopkins-Cole). The labile sulfur test is positive. Qualitative tests for phosphorus and for carbohydrate are negative. The nitroprusside test is negative. After reduction of a 1 per cent solution of prolactin with an equal volume of 5 per cent sodium cyanide, a weak but definitely positive nitroprusside reaction is obtained provided that cyanide reduction is permitted to proceed for approximately 2 hours at room temperature. Under comparable conditions, insulin was found to give a strongly positive sulfhydryl test within 5 minutes after the addition of the cyanide.

TYROSINE, TRYPTOPHANE, AND CYSTINE CONTENT OF PROLACTIN

Both crystalline and highly purified, amorphous prolactin preparations obtained from whole beef pituitary glands have been analyzed for tyrosine and tryptophane by the micro-method of Folin and Marenzi.[23] Cystine was determined by the method of Sullivan and Hess,[24] on an hydrolysate prepared by the HCl-formic acid procedure.[25] The results of these analyses are shown in TABLE 3 which, for comparison, also contains some analytical data from the literature.

[23] Folin, O., & Marenzi, A. D. Jour. Biol. Chem. 83: 89. 1929.
[24] Sullivan, M. X., & Hess, W. C. Pub. Health Rep. U.S.P.H.S. suppl. 86: 1930.
[25] Miller, G. L., & du Vigneaud, V. Jour. Biol. Chem. 118: 101. 1937.

It will be seen that the values obtained for the tyrosine, tryptophane and cystine content of prolactin are in good agreement for the 2 preparations employed. The tyrosine value found confirms that reported for beef prolactin by Li, Lyons, and Evans.[16c, 26] The tryptophane value obtained for prolactin is essentially the same (1.31 per cent) as that reported by Li, Lyons, and Evans,[26] who employed Lugg's[27] modification of the method of Folin and Ciocalteu.[25] More recently, however, these same workers have reinvestigated prolactin for its tryptophane content[16c] and report a value considerably higher (2.5 per cent) than that initially obtained.[26] The higher value is attributed to the fact that it was obtained by a glyoxylic acid method[29] which does not require hydrolysis of the protein, whereas the Lugg method originally employed for the determination of tryptophane involved an alkaline digestion which was believed to result in some destruction of the tryptophane.

TABLE 3

TYROSINE, TRYPTOPHANE, AND CYSTINE CONTENT OF BEEF PROLACTIN

Preparation	Tyrosine	Tryptophane	Cystine
	per cent	*per cent*	*per cent*
Purified amorphous prolactin	5.64	1.30	3.32
	5.50	1.27	3.41
Crystalline prolactin	5.42	1.34	3.34
	5.48	1.30	
Average*	5.51	1.30	3.36
Li, Lyons and Evans[26]	5.73	1.31	
Li, Lyons and Evans[16c]	5.7	2.5	
Fraenkel-Conrat[31]			3.0

* All values calculated on an ash- and moisture-free basis.

It is the opinion of the writer that Shaw and McFarlane,[29] who have studied the glyoxylic acid method in some detail, have not established that destruction of tryptophane occurs either under exactly the conditions of hydrolysis proposed by Folin and his colleagues[23,25] or in the procedure of Lugg.[27] Shaw and McFarlane have examined the effect of alkali and pressure on the stability of tryptophane in alkaline protein hydrolysates, but these are not the same hydrolytic conditions used in the Folin and Lugg procedures. Moreover, the glyoxylic acid method yields a tryptophane value for casein which is slightly lower than that

[26] Li, C. H., Lyons, W. R., & Evans, H. M. Jour. Biol. Chem. **136**: 709. 1940.
[27] Lugg, J. W. H. Biochem. Jour. **32**: 775. 1938.
[25] Folin, O., & Ciocalteu, V. Jour. Biol. Chem. **73**: 627. 1927.
[29] Shaw, J. L. D., & McFarlane, W. D. Canadian Jour. Research B **16**: 361. 1938; Jour. Biol. Chem. **132**: 387. 1940.

obtained by Folin and his colleagues, notwithstanding the fact that the latter investigators employed alkaline hydrolysis of the protein.

According to Lugg, tryptophane estimations require correction for 3 per cent loss if stannite has been included in the alkaline hydrolysis, or 6 per cent when alkali alone has been used.   Brand and Kassell,[30] in a careful and thorough study of the photometric determination of trypto-phane, tyrosine, diiodotyrosine and thyroxine, based on the procedure developed by Lugg from the Folin-Ciocalteu method, have suggested that no correction factor is required for the tryptophane content of alka-line hydrolysates of proteins.   In view of these above observations, the lower tryptophane values uniformly found in hydrolyzed as contrasted to unhydrolyzed proteins can hardly be attributed to the destruction of this amino acid during alkaline hydrolysis of the protein.   Rather, the disagreement appears to be based on the type of material to which colorimetric procedures are applied.

The cystine value found for prolactin is slightly higher than that re-cently reported by Fraenkel-Conrat.[31]   A cystine content of 3.36 per cent accounts for approximately 45 per cent of the total sulfur of prolactin.

EFFECT OF pH AND HEAT ON PROLACTIN ACTIVITY

Prolactin has been reported[5] to be relatively thermostable when heated in a boiling water bath for 1 hour at pH 7.0 to 8.0, but less stable at other pH ranges.   It is of course evident that many factors may influence the stability of protein hormones, e.g., the purity of the product and thus the presence of extraneous proteins which may increase the resistance of the hormone to labilizing agents.   In addition, protein and salt con-centrations and the pH of the solutions examined may have a profound influence on the stability of a biocatalytically active protein.

Solutions containing 0.04 per cent of highly purified, amorphous pro-lactin (30 I.U. mg.) were heated in a boiling water bath for 15 and 30 minute periods at pH values from 1 to 13.   The desired pH values were obtained with 0.01 N HCl and 0.01 N NaOH in suitable proportions.   At the end of the heating period, each sample was cooled immediately to room temperature, neutralized to approximately pH 7.0, made to a suitable volume and bio-assayed by the "local" intradermal method at a total protein level of 1, 10, and 100 μg.   The results are shown in TABLE 4 and graphically presented in FIGURE 7.   Even though the minimum effective dose has not been determined in these bio-assays, it is evident from the data that under the conditions employed prolactin is quite

30 Brand, E., & Kassell, B.   Jour. Biol. Chem. 131: 489. 1939.
31 Fraenkel-Conrat, H.   Jour. Biol. Chem. 142: 119. 1942.

TABLE 4

Effect of pH and Heat on Prolactin Activity

pH	Period of heating	Total dose injected for bio-assay		
		μg.[+]		
		1	10	100
	min.			
1	15	+	+	−
	30	0	+	−
3	15	+	+	−
	30	0	0	−
7	15	+	+	+
	30	0	0	+
9	15	+	+	+
	30	0	0	+
11	15	0	+	+
	30	0	0	+
13	15	0	0	+
	30	0	0	0

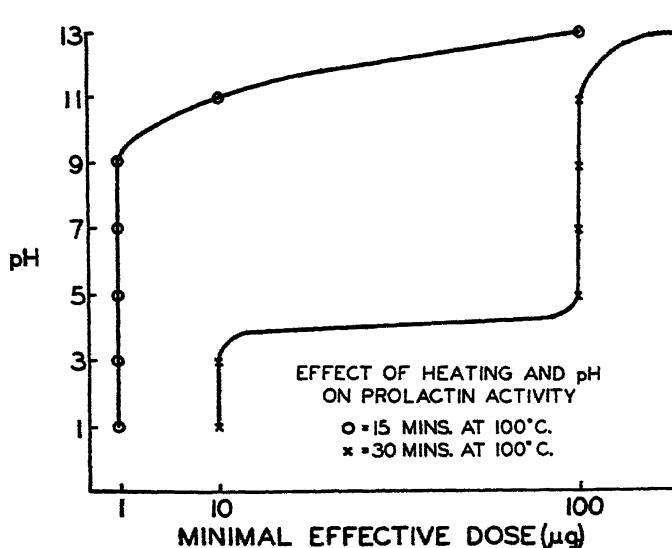* + = positive response—active; 0 = negative response—inactive.

FIGURE 7.   Effect of pH and heat on prolactin activity.

stable to heat in 0.04 per cent solutions of pH 1 to 9 when the heating is carried out for 15 minutes in a boiling water bath. At higher pH values there is a considerable loss of biological potency. With a 30 minute period of heating, there is a definite destruction of prolactin activity at all pH values, the effect again appearing to be most marked at pH 13. It appears, therefore, that prolactin may be classed as a heat-labile substance; this is in harmony with the protein nature of the hormone. The destruction of the hormone observed in the present experiments might find explanation in the splitting of labile sulfur from the hormone and in a significant degree of hydrolysis of the protein.

HYDROLYSIS OF PROLACTIN

Hydrolysis by Acid

Prolactin boiled for 18 hours with 20 per cent hydrochloric acid produces a hydrolysate which, after filtration and neutralization, gives no evidence of biological activity even though assays are conducted at a dose level approximately 400 times (based on the original protein concentration) that required to produce a positive response by the unhydrolyzed prolactin.

Digestion by Pepsin and by Trypsin

The prolactin used in the digestion experiments was active intradermally at a dose level of 0.5 μg. For the pepsin study, a 1:10,000 granular pepsin of the Wilson Laboratories was used. Digestion was conducted in 0.1N HCl at 37° C. The ratio of enzmye to prolactin was 1 : 250. After mixing the enzyme and prolactin solutions, samples were taken immediately and at 1, 2, and 3 hours of digestion time. Peptic action was stopped by making the samples slightly alkaline (pH 7.5) with 0.1N NaOH and permitting 15 minutes to elapse.

In the trypsin experiments, a commercial trypsin preparation (Fairchild) was employed. Digestion was studied at pH 7.8 in phosphate buffer. The ratio of trypsin to prolactin was 1:50. Samples were taken at zero time, and at $\frac{1}{2}$, 1, 2 and $3\frac{1}{2}$ hour periods. In order to stop further tryptic action in the samples taken for analysis, each was immersed in a boiling water bath for 1 minute, cooled rapidly and diluted to a convenient volume for assay. Bio-assays were conducted by the intradermal technique. The rate of protein digestion was followed as described by Northrop,[32] analyses being made for nonprotein nitrogen

[32] Northrop, J. H. "Crystalline Enzymes; the Chemistry of Pepsin, Trypsin, and Bacteriophage." Columbia Univ. Press. New York. 1939.

in filtrates obtained after precipitation of each 0.5 ml. sample with an equal volume of 10 per cent trichloroacetic acid.

The rate of hydrolysis of prolactin by pepsin and by trypsin is shown in FIGURE 8, and the results of the bio-assays are presented in TABLES
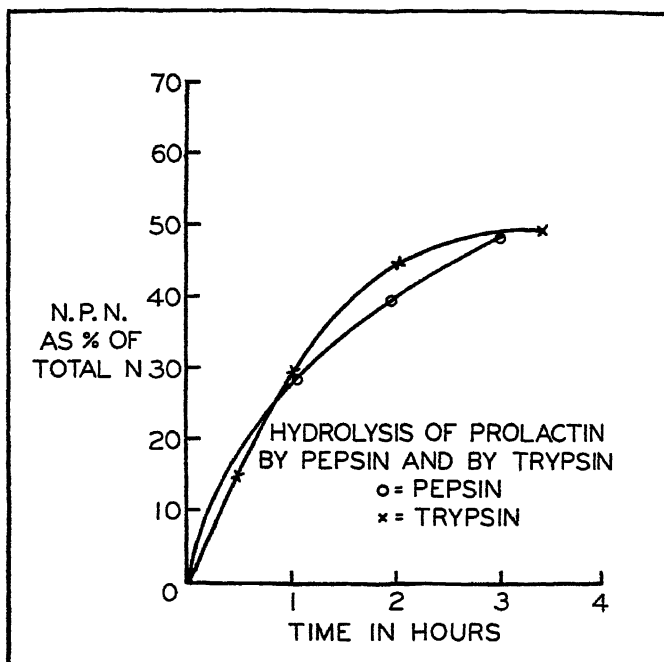
FIGURE 8.

5 and 6.   It will be seen that in the case of digestion with either pepsin or trypsin, approximately half of the original protein nitrogen is no longer precipitable by trichloroacetic acid after a 3-hour period of digestion. However, even though at the end of a 2-hour period there still remained in solution as much as 50 to 60 per cent of nitrogen which could be precipitated by trichloroacetic acid, the bio-assays show that this material was biologically inactive, even when given at dose levels from 100 to 120 times the amount necessary to elicit a positive prolactin response with the original prolactin preparation.   In other words, under the conditions of these enzyme studies, destruction of physiological activity occurs before there has been a hydrolysis of the protein into fragments which are no longer precipitable by trichloroacetic acid.

TABLE 5

RATE OF DIGESTION OF PROLACTIN BY PEPSIN AS CORRELATED WITH ALTERATIONS
IN BIOLOGICAL ACTIVITY OF THE HORMONE

Time	Per cent of original protein digested (non-precipitated by 10 per cent trichloroacetic acid solution)	Bio-assay. Total dose level μg.[†]		
hrs.				
0	0	1[*]	10[*]	100[*]
1	29	0.7	7[*]	70[*]
2	40	0.6	6	60
3	48	0.5	5	50

[†] All dosages, with the exception of those at 0 time, were calculated from nitrogen analysis.
[*] Active, i.e., positive prolactin response. All other assays were negative at the dose levels indicated.

TABLE 6

RATE OF DIGESTION OF PROLACTIN BY TRYPSIN AS CORRELATED WITH ALTERATIONS
IN BIOLOGICAL ACTIVITY OF THE HORMONE

Time	Per cent of original protein digested (non-precipitated by 10 per cent trichloroacetic acid solution)	Bio-assay. Total dose level μg.[†]		
hrs.				
0	0	1[*]	10[*]	100[*]
0.5	15	0.8[*]	8[*]	80[*]
1.0	30	0.7[*]	7[*]	70[*]
2.0	47	0.5	5	50
3.5	49	0.5	5	50

[†] All dosages, with the exception of those at 0 time, were calculated from nitrogen analysis.
[*] Active, i.e., positive prolactin response. All other assays were negative at the dose levels indicated.

The data obtained in the study of the enzymatic digestion of prolactin
are similar to those of Fisher and Scott[33] on the peptic hydrolysis of
insulin. These investigators observed that insulin activity was rapidly
destroyed by pepsin and, indeed, the hypoglycemic potency decreased
more rapidly than the rate of hydrolysis of the protein.

OTHER TYPES OF INACTIVATION

The preparation of a highly purified, biocatalytically active protein
is generally soon followed by efforts to determine whether particular
groups or groupings in the protein molecule are essential for the activity
which it manifests. It is to be hoped that studies of this type will, to
some extent by themselves, and perhaps even more when correlated with
similar experiments on other proteins, begin to lead to some understand-
ing regarding the fundamental underlying mechanism of imparting to a
particular protein a certain type of catalytic activity. The mechanism
of action of certain proteins which do not contain the usually well recog-

[33] Fisher, A. M., & Scott, D. A.   Jour. Biol. Chem. 106: 289.   1934.

nized prosthetic groups is one of the important unsolved problems of protein chemistry.

Inactivation with Ketene

The necessity of primary amino groups for the specific activity of the lactogenic hormone has been examined by investigators in the laboratory of Evans in California. In a preliminary note,[34] the acetylation of prolactin with ketene at 20° C. for 5 minutes is claimed to result in a 100 per cent acetylation of the amino groups of the hormone, accompanied by a complete physiological inactivation. These data are shown in TABLE 7

TABLE 7

ACETYLATION OF PROLACTIN BY KETENE

Type of preparation	Amino groups acetylated	Dose	Crop-sac reaction
	per cent	*mg.*	
Untreated	0	1.0	Pronounced
Acetylated at 0° for 5 min.	30	1.0	Negative to minimal
Acetylated at 20° for 5 min.	100	1.0	Negative
		4.0	Negative

and are taken from the communication of Li, Simpson and Evans.[34] It should be pointed out that in this study it has not been established that substitution of groups other than free amino groups has not occurred. This should be demonstrated for each protein studied, inasmuch as rates of substitution reactions with ketene for one protein are not necessarily valid for interpretation of data obtained in ketene experiments with other proteins.

It is of interest to compare these data for prolactin with the behavior of other well defined, biologically active proteins when treated with ketene. Whereas the practically complete acetylation of the free amino groups of pepsin,[35] insulin,[36] human chorionic gonadotropin[37] and, to a considerable degree, tobacco mosaic virus[38] by ketene does not diminish the biological activity of these proteins, diphtheria toxin,[39] the pituitary follicle cell-stimulating, interstitial-cell-stimulating[37] and lactogenic hormones,[34] and the gonadotropic hormone in pregnant mare serum[37] appear to require free amino groups for their activity.

[34] Li, C. H., Simpson, M. E., & Evans, H. M. Science 90: 140. 1939.
[35] Herriott, R. M., & Northrop, J. H. Jour. Gen. Physiol. 18: 35. 1934.
[36] Stern, K. G., & White, A. Jour. Biol. Chem. 122: 371. 1937–38.
[37] Li, C. H., Simpson, M. E., & Evans, H. M. Jour. Biol. Chem. 131: 259. 1939.
[38] Miller, G. L., & Stanley, W. M. Jour. Biol. Chem. 141: 905. 1941.
[39] Pappenheimer, A. M., Jr. Jour. Biol. Chem. 125: 201. 1938.

Inactivation with Nitrous Acid

Further evidence that the primary amino groups of the lactogenic hormone are essential for its specific activity has been claimed from a study of its behavior toward nitrous acid.[40]   Treatment of prolactin with nitrous acid for 30 minutes at zero degrees or at room temperature resulted in complete inactivation of the hormone.   No evidence establishing the mode of action of nitrous acid on prolactin is offered.   The oxidizing action of the reagent must be considered in making interpretations of changes in biocatalytic activity after treatment with nitrous acid.

Inactivation with Phenyl Isocyanate

In a preliminary communication, Bottomley and Folley[41] reported the preparation of a phenyl ureido derivative of prolactin by treatment of the protein with phenyl isocyanate at 0° C. and pH 8.0.   A loss of approximately 87 per cent of the biological activity was observed.   The data are taken as confirmation of the suggestion that the crop-stimulating activity of prolactin depends on the presence in the molecule of free amino groups.   It would seem advisable, however, in experiments involving the treatment of proteins with phenyl isocyanate, to demonstrate clearly by analysis that this reagent reacts *only* with the free amino groups of the particular protein being studied.   Miller and Stanley[3] have recently observed a marked reaction between phenyl isocyanate and the phenolic groups in the tobacco mosaic virus protein.   The reported inactivation of both prolactin[41] and of insulin[42] by phenyl isocyanate is open to other interpretations, particularly in view of the work of Miller and Stanley, and the demonstration, by the use of ketene, that the amino groups of insulin are not essential for the hypoglycemic action of the latter protein.[35]

Inactivation with Iodine

The reaction of iodine with lactogenic hormone has been studied in some detail by Li, Lyons, and Evans.[43]   Iodination in phosphate buffer (pH 7.0) at 20° C. for 1 hour resulted in an introduction of iodine into the phenolic residues of the tyrosine molecules of prolactin, and a complete inactivation of the hormone.

Inactivation with Thiol Compounds

Fraenkel-Conrat, Simpson and Evans[44] have recently described results obtained in a study of the effect of thiol compounds on the activity of the

0 Li, C. H., Lyons, W. R., Simpson, M. E., & Evans, H. M.   Science 90: 376.   1939.
1 Bottomley, A. C., & Folley, S. J.   Nature 145: 304.   1939.
2 Gaunt, W. E., & Wormall, A.,   Biochem. Jour. 30: 1915.   1936.
3 Li, C. H., Lyons, W. R., & Evans, H. M.   Jour. Biol. Chem. 139: 43.   1941.
1 Fraenkel-Conrat, H., Simpson, M. E., & Evans, H. M.   Jour. Biol. Chem. 142: 107.   1942.

lactogenic hormone.   Treatment of a solution of prolactin with a 40-fold concentration of cysteine transforms the hormone into an insoluble protein which, when redissolved under conditions which prevent autoxidation, is as biologically active as the untreated hormone.   When a 200-fold quantity of cysteine is employed to reduce prolactin, inactivation occurs.   Thioglycolic acid is approximately 50 times more effective than cysteine in causing inactivation of the lactogenic hormone.

COMMENT AND SUMMARY

Prolactin is the first anterior pituitary hormone to have been isolated in pure form.   The homogeneous nature of the preparations obtained is indicated from Tiselius electrophoresis experiments, solubility studies, and ultracentrifugal analysis.   All of the evidence available at the present time indicates the protein nature of the hormone; there is no indication of the presence in the molecule of any of the common types of prosthetic groupings.

The protein hormone has a molecular weight of approximately 32,000 to 35,000 and an isoelectric point at pH 5.65.   Some data for beef prolaction are shown in TABLE 8.   Prolactin may be classified as a heat-

TABLE 8
SOME DATA FOR BEEF PROLACTIN

Physiological activity	35 I. U. mg.
Carbon	51.50 per cent
Hydrogen	6.92 ” ”
Nitrogen	16.50 ” ”
Sulfur	2.00 ” ”
Tyrosine	5.7 ” ”
Tryptophane	1.3 ” ”
Cystine	3.4 ” ”
S_{20}	2.7 S
D_{20}	7.5×10^{-7}
Molecular weight	32,000–35,000
Isoelectric point	pH 5.65
Ultraviolet absorption maximum	2800 Å

labile protein.   Furthermore, reagents which produce even a mild degree of hydrolysis of the protein molecule cause a profound loss in the hormonal activity.   From studies directed toward an assay of the physiologically important functional groups in the prolactin molecule, it appears that substitution of the free amino groups or of the tyrosine phenolic groups of the protein results in a marked decline in, and usually a complete loss of, lactogenic potency.   Reduction of prolactin with an excess of cysteine or of thioglycolic acid also destroys biological activity.

The preparation of a pure hormone of the anterior pituitary gland will be of considerable aid in elucidating the complex physiology of this endocrine gland, and the rate of progress in this direction will be greatly accelerated as each additional anterior pituitary secretion is made available in a purified and homogeneous state. It is naturally of great interest to determine whether the prolactin preparations now available exhibit any other type of physiological activity which has been attributed to anterior pituitary extracts. Preliminary studies of this type have been conducted; the results are indicated in TABLE 9.

TABLE 9
BIO-ASSAYS OF PROLACTIN

Assay	Method	Result
Growth	Hypophysectomized rat	Negative
Thyrotropic	Day-old chick	"
Diabetogenic	Partially depancreatized rat	"
Adrenotropic	21-day-old rat	"
Gonadotropic	21-day-old mouse	"

It should be stressed that these are preliminary data and in each assay an amount of prolactin was administered which was approximately 100 times the amount of the protein of a whole beef pituitary gland saline extract (in terms of nitrogen times 6.25) which would yield a positive response in the test employed. These data are obviously not final. It is quite possible that similar tests at higher dose levels, or in other species, may indicate a multiple type of physiological activity for prolactin. The overlapping physiological behavior seen among the steroid hormones at high dose levels and under specially defined conditions of assay should be kept in mind. Indeed, data are already available indicating physiological activity for prolactin other than crop proliferation and initiation of lactation. Evans and his colleagues have recently reported evidence for two other types of physiological responses induced by this hormone. By means of the traumatic placentoma test in the rat, it has been demonstrated[45] that lactogenic hormone stimulates the production of progesterone by normally occurring or artificially induced lutein tissue. This interesting confirmation with purified prolactin of earlier suggestions that the lactogenic hormone is important in activation of the *corpus luteum* adds another example to the list of endocrine interrelationships. Equally striking is the recent claim from Evans' laboratory[46] that the lactogenic hormone increases the insulin content of the pancreas in nor-

[45] Evans, H. M., Simpson, M. E., & Lyons, W. R.   Proc. Soc. Exp. Biol. Med. 46: 586.   1941.
[46] Fraenkel-Conrat, H., Herring, V. V., Simpson, M. E., & Evans, H. M.   Am. Jour. Physiol 135: 404.   1942.

mal and in hypophysectomized rats. The data presented, however, do not appear significant when evaluated critically and require elaboration and confirmation by other investigators. This is particularly true in view of the fact that prolactin has also been reported to decrease pancreatic insulin.[47] The exact effect of prolactin on the insulin content of the pancreas should be determined by the use of experimental conditions rigidly established by Best and his colleagues at the University of Toronto.

These newer developments in the physiology of the lactogenic hormone, together with recent results[4b] obtained in collaboration with Dr. W. U. Gardner and discussed in the next section of this paper. are further emphasis of the fact that the nature of the physiological response manifested by a particular endocrine product may often be determined by the type of test animal and the dosage employed.

Section II: Mammogen

INTRODUCTION

The name, mammogen, was proposed in 1938 by Turner and his co-workers to describe the hormonal complex in the anterior pituitary which they believe is concerned with growth and development of the mammary gland. From experimental evidence at hand, the existence of two mammogen factors was postulated: one a duct growth factor, designated Mammogen I; and the other, named Mammogen II, believed to be responsible for completion of mammary development. causing growth of the mammary lobule-alveolar system.

EVIDENCE FOR THE EXISTENCE OF MAMMOGEN

The concept that the anterior pituitary gland exerts an important influence on the growth and development of the mammary gland has been generally accepted since 1936, when Reece, Turner, and Hill[49] reported that the mammary glands of completely hypophysectomized rats did not respond to the administration of ovarian hormones; a role of the anterior pituitary in the process of proliferation of mammary gland tissue seemed clearly indicated. This was further substantiated by the effectiveness of hypophyseal replacement therapy in hypophysectomized animals. The administration of crude anterior pituitary extracts, either alone or

[47] Funk, C., Chamelin, I. M., Wagreich, H., & Harrow, B.   Science 94: 260. 1941.
[48] Gardner, W. U., & White, A.   Proc. Soc. Exp. Biol. Med. 48: 590. 1941.
[49] Reece, R. P., Turner, C. W., & Hill, R. T.   Proc. Soc. Exp. Biol. Med. 34: 204. 1936.

with ovarian substance, to hypophysectomized animals of several species permitted normal growth and development of mammary glands.

Without presenting a detailed survey of the publications dealing with the problem of the existence of a pituitary mammogenic hormone, the conclusion is warranted that crude anterior pituitary extracts exert a profound influence on mammary growth.[50] The first experiments revealing the lactogenic activity of hypophyseal extracts were inadequate for the demonstration of mammary growth. Later experiments suggested a proliferation of the mammary glands of immature ovariectomized animals receiving pituitary extracts. This growth occurs in ovariectomized, in male or in hypophysectomized mice, or in rats following implantation of hypophyseal tissue or injection of hypophyseal extracts. Some investigators have associated this mammogenic activity with the protein components of such extracts and others with the lipid-soluble material of pituitary tissue.

Accepting the evidence that anterior pituitary gland tissue extracts contain a factor or factors which influence mammary development, and which have been termed mammogen by Turner and his colleagues, it is the purpose of the remainder of this discussion to consider three aspects of this problem: (1) the available information regarding the chemical nature of mammogen, (2) the relationship of mammogen to known hormones, and (3) the validity of the data supporting the mammogen hypothesis. Before passing to these topics, it may be of interest to mention briefly the methods devised for the assay of mammogen and mammary-stimulating materials.

METHODS OF ASSAY OF MAMMOGEN AND MAMMARY STIMULATING MATERIALS

In their studies of mammogen, Turner and his colleagues have employed the mouse as the assay animal. The activity of the anterior pituitary duct-growth factor (Mammogen I) has been measured by its effect on the mammary duct system of male mice. The biological assay of the anterior pituitary lobule-alveolar growth factor (Mammogen II) has been conducted in castrate, virgin female mice given small amounts of estrone which appears to enhance the activity of the pituitary lobule-alveolar growth factor. Other investigators have preferred to use hypophysectomized animals in order to eliminate the influence of the test subject's own pituitary and also to permit the evaluation of various extracts in terms of effectiveness as replacement therapy.

[50] **Turner, C.W.** in "Sex and Internal Secretions," 2nd edit. E. Allen, Ed. Williams and Wilkins Co. Baltimore. 1939. p. 740. (Complete review of literature on the mammary gland.)

CHEMICAL PROPERTIES OF MAMMOGEN

Preparations Soluble in Lipid Solvents

The first report of the full development of the mammary glands in experimental animals injected with anterior pituitary preparations was made in 1930 by Corner,[31] who employed an alkaline extract of sheep anterior pituitary glands. He stated that ether extraction of this aqueous solution appeared to remove the mammary growth-promoting activity. The ether-soluble fractions, however, exhibited variable physiological potency; the active principle was rapidly destroyed on heating for 1 hour at 90° C. and appeared to deteriorate rapidly on storage.

The bulk of the evidence that the mammogenic factors in the anterior pituitary are soluble in lipid solvents has been derived from the investigations of Turner and his colleagues. Following their development of a suitable assay technique for the duct growth component of mammogen, Lewis and Turner[52] studied the chemical characteristics of the factor and concluded that this hormone is soluble in lipid solvents and is distinct from other known pituitary hormones. The evidence on which this conclusion was based may be briefly summarized (potency refers to activity in promoting duct growth in the rudimentary mammary glands of young male mice):

(1) Desiccation of fresh anterior pituitary lobe tissue from pregnant cattle with acetone and ether results in a 62 per cent loss of potency.

(2) The extraction of acetone-dried pituitary glands by a procedure (60 per cent alcohol at pH 9.0 to 10.0) which removed most of the lactogenic, thyrotropic, carbohydrate metabolism, and gonadotropic potency of fresh pituitary tissue[53] leaves an insoluble residue which is active. It should be pointed out, however, that precipitates prepared from the initial extracts by the addition of 3 volumes of 95 per cent alcohol at pH 5.7 also showed, after drying with alcohol and ether, a definite but smaller degree of potency.

(3) Residues obtained by evaporation of the 87 per cent alcohol solution used to precipitate the initial extract gave strongly positive results.

(4) Similar experiments with fresh, anterior pituitary tissue also yielded alcoholic solutions, after precipitation of the protein hormones in the usual manner, which on evaporation at low temperature *in vacuo* left highly potent residues. However, direct extraction of ground an-

[31] Corner, G. W. Am. Jour. Physiol. **95**: 43. 1930.
[52] Lewis, A. A., & Turner, C. W. Mo. Agr. Exp. Sta. Res. Bull. No. 310. 1939.
[53] Bergman, A. J., Houchin, O. B., & Turner, C. W. Endocrinology **24**: 547. 1939.

terior pituitary tissue with several volumes of 95 per cent alcohol did not yield active products.

(5) Extraction with several volumes of ether-alcohol mixture (1:3) at 50° C. was very efficient in removing mammogen. Removal of the solvent at reduced pressure yielded an active residue. The latter was fractionated by ether extraction at room temperature. The ether-soluble fraction is highly active; the ether-insoluble material is inactive. The best product reported to the present time was obtained in this manner. This preparation gave a positive response in 11 of a group of 14 mice at a dosage of 0.25 mg. per mouse.

Evidence that two distinct mammogen factors are concerned with growth and development of the mammary gland has also been reported by Turner and his colleagues. Fresh anterior pituitary tissue containing mammogen produced both duct development and lobule hyperplasia, whereas the lipid extracts caused only duct development.[32,54]

There have as yet been little data available regarding the chemical nature of mammogen, other than the claim of solubility in lipid solvents at pH ranges in which pituitary protein hormones are precipitable. This behavior is said to differentiate mammogen from other known anterior pituitary hormones. Lewis and Turner[52] reported that mammogen is quite unstable to heat and possibly to oxidation. The heat lability of mammogen has been stressed as a property which distinguishes this hormone from estrogenic substances. The active lipid extracts deteriorated rapidly if not kept at low temperatures.

ANTERIOR PITUITARY PROTEIN HORMONES IN RELATION TO MAMMARY GROWTH

Before discussing further the suggestions of Turner and his colleagues that the anterior pituitary secretes a hormonal complex, mammogen, which is distinct from other known anterior pituitary hormones, it may be advisable first to indicate the established roles of certain anterior pituitary protein hormones in the growth of the mammary gland. Furthermore, experiments will be described in which growth and development of the mammary gland in hypophysectomized animals are produced with already known and highly purified hormonal preparations. In view of these observations, it may not be necessary to postulate a new anterior pituitary complex, mammogen, as a regulatory factor required for mammary gland growth, and it then becomes permissible to re-evaluate previous experimental results in the light of the newer data.

The anterior pituitary hormones whose chemical nature has been

[54] Mixner, J. P., Lewis, A. A., & Turner, C. W. Endocrinology **26**: 888. 1940.

studied in more or less detail appear to be proteins. Although a number of negative results have been reported in early investigations, it is now clearly established that crude aqueous extracts of anterior pituitary tissue contain a factor, or factors, which cause mammary gland development in a wide variety of different species, both in normal and in hypophysectomized test animals. Although Corner[51] and later Turner and his colleagues[52] have, as already discussed, associated the pituitary factor with material soluble in lipid solvents, some investigators have also reported positive results with crude anterior pituitary protein fractions. The activity of these products is generally enhanced by the simultaneous administration of estrogen, although the latter is not always essential when crude pituitary extracts are employed. Asdell and his colleagues[55] reported that extracts of anterior pituitary gland of the sheep prepared by extraction with sodium hydroxide were found to cause mammary growth and secretion in the virgin female ovariectomized rabbit. Acid extracts were without effect on mammary growth, but induced lactation in the dry parous ovariectomized rabbit. This action was due to the prolactin in the acid extract. These data were interpreted as suggesting that the mammary growth and secretion effects are due to separate substances.

Greep and Stavely,[56] in a recent effort to confirm the presence of a lipid-soluble mammogen in the pituitary gland, assayed the warm (50° C.) alcohol-ether soluble material from both desiccated and fresh pituitary glands. Whereas the desiccated and powdered pituitaries, injected in suspension in saline, produced unquestionable mammary growth in doses equivalent to 50 mg. of fresh gland, the administration of lipid-soluble residues in doses equivalent to as much as 3200 mg. of fresh gland failed to induce mammary development. This was true of both types of alcohol-ether extracts, i.e., whether made from desiccated pituitary tissue or from the fresh gland. On the other hand, Greep and Stavely reported marked duct growth in the test animals given the alcohol-ether-insoluble tissue residue in doses equivalent to 200 mg. of fresh gland.

The observations of Greep and Stavely that mammary growth factor is demonstrable in the protein fraction of pituitary tissue, and not evident in lipid extracts, make advisable a re-evaluation of the role which the known pituitary hormones may play in mammary gland growth. Lyons and Catchpole[57] in 1933 stated that a mammary gland in a virgin female

[55] Asdell, S. A., Brooks, H. J., Salisbury, G. W., & Seidenstein, H. R.   Cornell Univ. Agr. Exp. Sta. Mem. 198.   1936.
[56] Greep, R. O., & Stavely, H. E.   Endocrinology **29**: 18.   1941.
[57] Lyons, W. R., & Catchpole, H. R.   Proc. Soc. Exp. Biol. Med. **31**: 299.   1933.

rabbit. normally matured and then castrated, will respond by growth of ducts and alveoli and lactation to administration of pituitary lactogenic extract. It is interesting to note, in view of later developments, that these investigators stated their belief that if significant amounts of ovarian hormones remained in the body after castration, these hormones could act only by synergistic stimulation with the lactogenic hormone. In 1936, Lyons[58] reiterated his view that lactogenic hormone could induce alveolar hyperplasia by demonstrating secretion and alveolar proliferation following injection of prolactin into young male rabbits previously treated for a period of 3 weeks with daily injections of estrogen. In a footnote appearing in a recent publication of Evans, Simpson and Lyons,[45] however, it is stated that as much as 10 mg. of purified lactogenic hormone have been injected into both normal mature and hypophysectomized female rats without stimulating the proliferation of mammary lobules. Lyons and his colleagues[59] have corrected the observation in the above referred footnote and report that the injection of prolactin into young, normal female rats produces excellent mammary development. This mammary gland growth is attributed to the combined action of estrin and progestin.

In 1937, Gomez and Turner[60] reported their inability to demonstrate the growth of the duct system of the mammary glands of completely hypophysectomized rats and guinea pigs treated with purified thyrotropic, adrenotropic, or lactogenic hormones alone and in combination with estrogen and progesterone. Desiccated whole sheep pituitaries were also without effect under similar experimental conditions. In view of the recent demonstration by Greep and Stavely[56] that desiccated beef pituitaries produce definite mammary growth, and the observations of Gardner[61] that the mammary glands of hypophysectomized male mice showed development when estrogen and progesterone were injected, the negative findings of Gomez and Turner with similar preparations make necessary a re-examination of the effects of individual pituitary protein hormones which have been reported to be without stimulative action on the mammary gland.

Moreover, the striking demonstrations of the local action of estrogens on mammary development in a variety of species throw further doubt on the absolute necessity of the pituitary in the experimental development of the mammary glands and rather emphasize the observations on estrogen and progesterone, which Gomez and Turner claimed were with-

[58] Lyons, W. R.   Anat. Rec. 64: 31, suppl.   1936.
[59] Lyons, W. R., Simpson, M. E., & Evans, H. M.   Proc. Soc. Exp. Biol. Med. 48: 654.   1941.
[60] Gomez, E. T., & Turner, C. W.   Mo. Agr. Exp. Sta. Res. Bull.   No. 259.   1937.
[61] Gardner, W. U.   Proc. Soc. Exp. Biol. Med. 45: 835.   1940.

out effect on mammary proliferation. For example. Gardner and Chamberlin[62] in a recent study with male mice have reported that estrone in alcoholic solution when applied to one or more areas of the non-epilated skin produces growth of only those glands near the site of application of the estrone. Such experiments indicate that the response of the mammary tissue *in vivo* is directly stimulated through the action of estrogen. If the response of the glands to estrogen were necessarily mediated by a pituitary factor, all of the glands should have responded as well as did those to which the hormone was applied. In the hypophysectomized rat,[63] however, treatment with estrogen on the skin over the mammary gland is without effect, although in the normal or partially hypophysectomized rat, new growth is stimulated. These experiments are interpreted as further evidence that a pituitary mammogen is essential for steroid hormone effects on the mammary glands of hypophysectomized rats. It may be granted that a known pituitary factor affects mammary gland growth, without postulating a new mammogen complex. This is particularly true in view of the role of prolactin in stimulating mammary development.[48] These experiments will be considered later. The failure of the hypophysectomized rat to respond to combinations of desoxycorticosterone and estrogen[63] is in contrast to the behavior of the hypophysectomized mouse which exhibits definite mammary growth following treatment with these hormones.[61] It would have been of considerable interest if Leonard and Reece[63] had administered progesterone together with estrogen to their hypophysectomized rats. This combination of steroid hormones is effective in the hypophysectomized mouse, and had similar results been obtained with the rat, the ineffectiveness of local application to mammary tissue in hypophysectomized rats might then be attributed to the absence of prolactin and the capacity of the latter hormone to stimulate the production of progesterone.[45] It may also be pointed out that mammary development has been reported[55] in hypophysectomized rabbits injected with estrogen and progesterone. This species, therefore, behaves in a manner similar to the mouse in its response to a combination of these two steroid hormones.

Reece and Leonard[64] have recently published data contributing to the subject of the effects of pituitary protein hormones on the mammary gland. Hypophysectomized rats given estrogen and gonadotropic hormone prepared either from pregnancy urine or from gland sources exhibited no mammary proliferation. On the other hand, the mammary glands of experimental animals receiving a growth hormone preparation

62 Gardner, W. U., & Chamberlin, T. L. Yale Jour. Biol. Med. 13: 462. 1941.
63 Leonard, S. L., & Reece, R. P. Endocrinology 30: 32. 1942.
64 Reece, R. P., & Leonard, S. L. Endocrinology 29: 297. 1941.

plus estrogen showed a stimulated growth of the duct system. It is interesting to note that the growth hormone preparation alone produced slight mammary growth. The growth hormone used contained thyrotropic hormone and probably small amounts of adrenocorticotropic substance. Gonadotropic hormone was also present in the growth preparation but the effect of the former can be disregarded in view of the negative findings with purified gonadotropic substances. The results obtained by Reece and Leonard were interpreted by those authors in terms of the effect of the mammogenic factor contained in the growth hormone preparation.

In collaboration with Dr. W. U. Gardner, of the Department of Anatomy of the Yale University School of Medicine, it has been possible recently to examine the influence of certain purified pituitary protein hormones on mammary growth in hypophysectomized male mice receiving estrogen. Gardner[61] has demonstrated that the mammary glands of hypophysectomized male mice may be developed slightly by the administration of estradiol propionate. progesterone, or desoxycorticosterone. A more extensive and more rapid proliferation of the mammary ducts of hypophysectomized mice occurred when desoxycorticosterone acetate or progesterone was injected together with the estrogen. Highly purified lactogenic hormone, prepared in our laboratory and shown to be homogeneous by solubility studies, electrophoretic behavior, and ultracentrifugal analysis was available, and this preparation has been tested for mammary-growth stimulating effects in hypophysectomized male mice receiving estrogen. Several other prolactin preparations were also studied in the following manner. Hybrid male mice from 4 to 8 weeks of age were hypophysectomized by means of the procedure described by Thomas.[65] The mice were maintained on a stock diet of Fox Chow and water. Completeness of operation was checked by examination of serial sections of the sella. From 3 to 47 days after operation, injections of lactogenic hormone with or without estrogen were started; the prolactin was administered intraperitoneally daily and the estrogen[66] subcutaneously every other day. On the 10th to 13th day the animals were killed and the mammary glands studied from stained and dissected preparations. Untreated hypophysectomized mice served as controls. Preliminary experiments have also been conducted with other types of extracts and hormonal preparations.

Some of the data obtained have already been published.[48] Since the time of the latter publication, experiments have been extended and a

summary of the results are shown in TABLE 10. Five mice received only a commercial preparation of prolactin (SC) which contained 12.5 I.U. per mg. A slight mammary growth occurred in 3 of these animals. Two of the latter were caged with animals receiving estrogen and may have acquired enough of the latter hormone by contact to elicit some mammary response. A moderately extensive mammary growth occurred in all mice receiving both estrogen and the same preparation of prolactin.

The rudimentary glands were not stimulated in the 12 operated animals receiving either of the 2 preparations of highly purified prolactin (SP and AW) at any of the 3 different dose levels. The failure of mammary growth to occur in hypophysectomized male mice given purified lactogenic hormone is in agreement with the observed lack of growth of the mammary glands in hypophysectomized rats injected with large amounts of prolactin.[45] The 13 hypophysectomized mice receiving estrogen in addition to the purified lactogenic preparations showed mammary growth rated as $++$ in 11 animals and $+$ in the other 2. At the

TABLE 10

MAMMARY RESPONSES IN HYPOPHYSECTOMIZED MALE MICE RECEIVING VARIOUS HORMONES AND EXTRACTS ALONE AND TOGETHER WITH ESTROGEN

Number of Mice	Treatment*	Period treated	Number of mice showing mammary growth
		Days	
13	Saline pituitary extract	9–16	3
17	Saline pituitary extract + estradiol dipropionate	4–16	17
5	Prolactin⁻ˢᶜ (Schering—12.5 I.U. mg.)	11–12	3
5	Prolactin⁻ˢᶜ (Schering—12.5 I.U. mg.) + estradiol dipropionate	11–12	5
8	Prolactin⁻ˢᶠ (Schering—35 I.U. mg.)	11–12	0
12	Prolactin⁻ˢᵖ (Schering—35 I.U. mg.) + estradiol dipropionate	9	12
4†	Prolactin⁻ᴬᵂ (35 I.U. mg.)	10	0
1	Prolactin⁻ᴬᵂ (35 I.U. mg.) + estradiol dipropionate	10	1
5	Saline fetal extract (mouse)	9–12	0
2	Saline fetal extract (mouse) + estradiol dipropionate	9	0
2	Thyrotropic hormone	9	0
3	Thyrotropic hormone + estradiol dipropionate	9	0
2	Thyrotropic hormone + prolactin	9	0
2	Thyrotropic hormone + prolactin + estradiol dipropionate	9	2

* Estradiol dipropionate, 1 μg. every other day. Pituitary hormones given daily. Prolactin given at dose levels of 0.1, 0.3, and 1.0 mg. daily with uniform results. Thyrotropic hormone given in doses of 0.25 mg. daily. Saline pituitary extract given in doses of 0.25 mg. total solids daily. Saline fetal extract given in doses of 0.2 to 2.2 mg. total solids daily.

† This prolactin preparation has been demonstrated to be homogeneous. White, A., Bonsnes, R. W. & Long, C. N. H. Jour. Biol. Chem. 143: 447. 1942.

time they were removed the glands were growing rapidly as indicated by the enlarged end-buds containing numerous mitotic figures. The extent of mammary proliferation averaged somewhat less than that obtained in unoperated mice receiving estrogen, and was comparable to that induced in hypophysectomized mice receiving estrogen and progesterone.[61]

It has further been observed in these studies that a highly purified preparation of pituitary thyrotropic hormone, containing significant amounts of gonad-stimulating material, failed to produce mammary gland growth in hypophysectomized male mice receiving estrogen. The inability of these two pituitary hormones to stimulate mammary growth under the experimental conditions employed confirms the observations of Reece and Leonard[64] with hypophysectomized rats. Three of 13 hypophysectomized male mice receiving saline extracts of pituitary tissue showed mammary growth. All mice given estrogen and the saline extract showed mammary growth comparable to that in mice receiving estrogen and prolactin.

Further evidence that prolactin is the limiting factor in mammary development in these studies is seen in the experiments in which thyrotropic hormone and estrogen, either alone or in combination, did not induce mammary growth, whereas the addition of prolactin to these two other hormones produced mammary proliferation.

THE PRESENT STATUS OF THE MAMMOGEN HYPOTHESIS

In a discussion of the question of the existence of a specific anterior pituitary mammogen factor or factors regulating the development of the mammary gland, it is obviously necessary to consider the following questions:

(1) Can an adequate explanation of mammary development be made which is based on known hormonal factors without the inclusion of the mammogen hypothesis?

(2) In the event that the answer to the first question is an affirmative one, can the experimental data supporting the mammogen hypothesis be rationalized from the information available, disregarding the postulation of new hormones?

(3) In the event that the answer to the first question is affirmative, and to the second negative, is it justifiable to assume that there may be several different anterior pituitary hormones controlling the development of the mammary gland?

The experiments which have been described with prolactin demonstrate for the first time that highly purified lactogenic hormone admin-

istered with estrogen to hypophysectomized male mice will produce mammary growth in these animals. Inasmuch as the prolactin alone has no effect, and the injection of estrogen into hypophysectomized animals produces no marked mammary stimulation, it is evident that the action of both of these hormones together is required. The conclusion may be drawn that prolactin may either sensitize the mammary tissue of hypophysectomized mice to estrogen or improve the general condition of these animals so that mammary proliferation may occur. Whatever the correct interpretation of the results may be, the need for both the pituitary and the ovarian factors in these experiments seems evident. It should be kept in mind, however, that experimentally the pituitary factor may be replaced by either progesterone or desoxycorticosterone acetate, since either of the latter steroids will produce mammary growth in hypophysectomized male mice treated with estrogen.[61] Moreover, the nonessential role of the pituitary has been emphasized by the production of mammary development by direct application of estrogen to the skin. This is not to be construed, however, as minimizing the importance of the pituitary gland or its secretions in normal mammary gland physiology.

The preceding discussion pertains to evidence obtained in experimental animals. It is quite obvious that a number of endocrine glands, i.e., the pituitary, the adrenals, and the gonads, and other factors may contribute to complete development of the mammary gland. It is also obvious that inasmuch as under normal circumstances the secretion of adrenal cortical steroidal substances is under the influence of the anterior pituitary, pituitary factors other than prolactin may influence mammary development. Yet another possibility has little experimental support, namely, that prolactin itself may influence adrenal cortical activity.

Although it is true that previous experiments with prolactin and estrogen have yielded negative results in mammary growth studies, the body of positive evidence previously obtained with crude and now with highly purified prolactin permits the conclusion that an adequate explanation of mammary development may be made on the basis of the effects of combinations of several known hormones, e.g., prolactin and estrogen or progesterone and estrogen, on the mammary gland.

With the first proposed question now answered in the affirmative, we come to the second, and somewhat more difficult one, namely, the rationalization of the experimental data supporting the mammogen hypothesis solely on the basis of experimental conclusions which do not include the existence of the mammogen complex.

The evidence for the presence in anterior pituitary tissue of a lipid-

soluble factor which produces growth of the mammary gland must, to be accepted, preclude several other possible explanations of the data. One of these necessitates an examination of the methods of preparing mammogen to ascertain whether the extraction methods would remove any of the known anterior pituitary hormones, *e.g.*, the proteins and more specifically, prolactin. The use of organic solvents in which proteins are generally insoluble would appear to limit this possibility. It is true, however, that the extraction of fresh pituitary tissue with organic solvents is essentially an extraction with a mixture of water and organic solvent, and the extractability of the pituitary protein hormones with aqueous alcohol and aqueous acetone is very great indeed. The fact that such extracts are subsequently increased in organic solvent concentration to as much as 80 per cent, in terms of alcohol or acetone, and at a pH at which the anterior pituitary protein hormones exhibit minimum solubility, does not permit one to ignore the definite and significant solubility of certain of these proteins under these conditions. Concentration of supernatants, after removal of precipitated proteins, may therefore serve to concentrate small amounts of pituitary protein hormones in these solutions.

Notwithstanding the above intimation that there may be certain anterior pituitary proteins in mammogen preparations, one of which is now established as functioning in mammary development, the technique used by Turner and his colleagues for further concentration of mammogen would appear to eliminate an explanation of their data on the basis of the presence of pituitary protein hormones in mammogen preparations. Lewis and Turner[52] reported that oily residues, obtained by the evaporation of aqueous-organic or organic solvent extracts of pituitary tissue or of supernatants remaining after precipitation of pituitary proteins, could be further purified by means of ether extraction. Moreover, the mammogenic activity was in the ether-soluble fraction; the ether-insoluble residue gave negative results on assay for mammogen. In view of these latter observations, the presence of protein material in the active mammogen preparations seems unlikely, although the solubility of proteins in lipid solvents, in the presence of lipoidal material, is not unknown.

Let us now turn to an examination of the possibility that known lipid-soluble hormones may be present in the pituitary gland and may account for the effectiveness of mammogen preparations in mammary development. The mammary growth-promoting action of estrogen, progesterone and adrenal cortical hormones makes valid this inquiry. Although steroid hormones have not been isolated in pure form from pit-

uitary tissue, there is ample physiological evidence for their presence in significant amounts. Brouha and Simonnet,[67] in 1927, reported that the subcutaneous administration of 20 mg. of lipid-soluble extract of cattle pituitaries into immature rats induced vaginal opening and cytological changes of the vagina characteristic of the estrus cycle and development of the uterus. In adult female castrate rats 50 to 100 mg. of material resulted in a complete estrus cycle. Callow and Parkes[68] obtained similar results in 1936 when they observed that an acetone extract of the hypophyses of cattle proved to be highly estrogenic. Moreover, Lewis and Turner[52] have investigated the possible estrogenic potency of fresh anterior pituitary tissue and extracts, using vaginal smear reactions in ovariectomized mature mice and increase of uterine weight in immature mice as criteria of estrogenic potency. Approximately 17 per cent of the test animals showed estrus vaginal smear reactions and the presence of a small amount of estrogen was indicated by the uterine weight test. It should be pointed out, however, that the majority of the positive responses were obtained with a single preparation, whereas each of 4 other extracts gave negative vaginal smears.

Qualitative examination of anterior pituitary tissue and extracts having mammary growth activity for the presence of progesterone has recently been reported by Turner and his colleagues.[69] This seemed highly desirable in view of the observation of Gardner and Hill[70] that progesterone alone stimulates the growth of the duct system of the mammary gland. But the Missouri group found that amounts of fresh pregnant cattle pituitary which will stimulate growth of the lobule-alveolar system in castrate female mice did not contain sufficient progesterone to give a positive response by the sensitive McGinty technic. Lipid extracts of the anterior pituitary which stimulated duct growth in the male mouse were also found to be negative for progesterone. These observations were taken to indicate that the mammogenic effects of the anterior pituitary are not due to progesterone. It should not be overlooked, however, that the presence of other sex hormones in these extracts may inhibit the progesterone response.

In view of the demonstrated presence of estrogenic hormone in the pituitary, and its occurrence in the lipid concentrates employed by Turner and his collaborators, and inasmuch as normal male and castrate female mice have been the assay animals for demonstration of mammogen activity, it is evidently possible that the functioning of the pituitary in

[67] Brouha, L., & Simonnet, H.   Comp. rend. Soc. Biol. **96**: 1275.   1927.
[68] Callow, R. K., & Parkes, A. S.   Jour. Physiol. **87**: 288.   1936.
[69] Trentin, J. J., Mixner, J. P., Lewis, A. A., & Turner, C. W.   Proc. Soc. Exp. Biol. Med. **46**: 440.   1941.
[70] Gardner, W. U., & Hill, R. T.   Proc. Soc. Exp. Biol. Med. **34**: 718.   1936.

these mice supplied one of the pituitary hormones, *e.g.*, prolactin, whose synergistic effects with estrogen on mammary gland development has now been established.   However, in a recent publication Lewis, Gomez, and Turner[71] have compared mammary gland development with mammogen in the castrate and in the hypophysectomized rat.   It was demonstrated that lipid extracts of anterior pituitary glands from pregnant cattle produce mammary duct growth in both castrate and in hypophysectomized rats.   The castrate animals were males of varying ages: the hypophysectomized animals were young male and female rats. There was no significant change in the weights of the thyroids, ovaries and uteri of the treated hypophysectomized female rats as compared with similar uninjected controls.   Adrenal gland weights of treated rats appeared to be somewhat smaller than those of the control group of animals.

The demonstration of the effectiveness of lipid pituitary extracts in producing mammary growth in hypophysectomized rats appears to rule out a pituitary influence in these animals, particularly in view of the already discussed improbability of the presence of protein pituitary hormones in these extracts.   But the demonstrated capacity of certain steroid hormones to produce mammary growth must be kept in mind. TABLE 11 is compiled from data in the publication of Turner and his colleagues.   It will be observed that negative results on mammary duct growth were obtained in castrate male rats given Mammogen I in a total dose of 1.8 m.u. (mouse units) in 6 injections.   But when larger total doses were given for longer period of time (0.18 to 9.0 m.u. of Mammogen I daily for 28 to 30 days), positive duct-end buds and considerable duct development was seen at the higher dosages.   The percentage response was not high until several mouse units a day were given for 16 to 30 days.   The lipid extracts employed are reported to have an activity of 1 Mammogen I mouse unit in 0.1 to 1.0 mg. of material.   In other words, an animal given 0.5 mouse units daily may have received from 0.05 to 0.5 mg. daily, depending on the activity of the particular preparation employed.   Over a period of average length of 23 days, the total amount of material injected would have varied from 1.2 to 12.0 mg. In the experiments with hypophysectomized rats, 4 to 16 m.u. were given daily for 7 to 10 days.   This is an average total dose of approximately 8 to 80 mg. per animal, depending on the activity of the particular mammogen preparation employed.   Similar relatively large dosages have been used in previous experiments from Turner's laboratory demonstrating a lipid-soluble mammogen.   If 0.1 per cent of the lipid-soluble

[71] Lewis, A. A., Gomez, E. T., & Turner, C. W.   Endocrinology 30: 37.   1942.

TABLE 11

EFFECT OF MAMMOGEN I EXTRACT ON THE MAMMARY GLANDS OF CASTRATE AND OF HYPOPHYSECTOMIZED RATS[71]

Type of rat	Number of animals	Dose per day (mouse units)	Number of injections	Total dose (mouse units)	Mammary change
Male, castrate	3	untreated	0		Regression or no change
"	3	1.8 cc. olive oil	6		Regressed
"	8	0.003 0.03	6	0.018 0.18	No duct development
"	9	0.07-0.3	6	0.12-1.8	Appeared active; no end buds
"	18	0.006-0.3	30	0.18-0.0	5 positive; end buds
"	9	2.0-8.0	28	46-224	3 positive; end buds
Male, hypophysectomized	3	0			Ducts atrophic
"	4	4 16	7 10	28 160	Positive
Female, hypophysectomized	11	0			Ducts atrophic
"	12	1 16	10	40 160	Positive

material consisted of steroid hormone substances, the variable positive mammary growth observed might be expected.

The possible general occurrence of the steroid hormones in small amounts in various tissues, and their effect in very small dosages on the mammary gland must be kept in mind when evaluating results which are interpreted as indicating the existence of new specific factors concerned with mammary gland development.   Furthermore, Nelson[72] has recently presented additional physiological evidence demonstrating that sex hormones are produced in the adrenal cortex.   These experiments supplement the isolation experiments of Reichstein, of Wintersteiner, and of Pfiffner, and studies by others of the urines of patients with pathological changes of the adrenals.   The latter endocrine organs, on the basis of already available evidence, may assume an important role in mammary gland physiology.   It may also be pointed out that Lewis and Turner[32] were able to cause mammary hyperplasia in male mice with lipid-soluble extracts of guinea pig livers, although it is true that 80 to 90 mg. of material were required to produce a positive response in 30 per cent of the animals used.

In summing up the present available evidence in an effort to obtain an answer to the second proposed question, namely, can the data supporting the mammogen hypothesis be rationalized from this evidence (disregarding the postulation of new hormones) the following points may be made:

(1) The wide distribution of varying quantities of steroid hormones in the body tissues, and the synergistic and broad physiological actions of certain of these hormones, make it necessary to exclude completely their possible presence in any crude extracts alleged to contain new lipid-soluble hormonal factors.   This is particularly true when:

    (a) Small amounts of certain known steroid hormones are demonstrable in lipid extracts of various tissues.

    (b) Relatively large quantities of lipid-soluble fractions are required to produce a given mammary gland response in studies designed to support claims for new factors.

(2) It has been established that certain endocrine organs, e.g., the adrenal cortex, produce several types of physiologically active steroids (estrogens), some of which may have a main site of production elsewhere in the body.   Under these circumstances, removal of the principal locus of formation of a particular hormone does not completely eliminate this hormone from the physiological picture.

(3) The data which have been presented in support of the mammogen

72 Nelson, W. O.   Anat. Rec. 81: 97.   Suppl.   1941.

hypothesis may find explanation in the established roles of certain hormones, *e.g.*, prolactin, estrogens, progesterone, and desoxycorticosterone, in mammary gland growth and development, and the possible but as yet unproven roles of other hormones, *e.g.*, corticosterone, adrenotropic hormone of the anterior pituitary, in these processes.

4) In view of the foregoing, it is concluded that the existing evidence for the secretion of a lipid-soluble mammogen complex by the anterior pituitary gland must be supplemented by: (a) purification of and more intimate chemical information regarding the nature of the lipid-soluble extracts of this tissue, and (b) a more highly refined experimental elimination of possible influences of known hormonal factors in physiological experiments designed to obtain evidence for the mammogen hypothesis.

THE GROWTH AND METABOLIC HORMONES
OF THE ANTERIOR PITUITARY*

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INTRODUCTION

The discussion of the anterior pituitary factors that influence the growth and metabolism of animals is one of unusual complexity. In the first place, it is obviously impossible to distinguish between effects described as "growth promotion" and those that are termed effects on metabolism, since in the last analysis growth in the true sense is dependent on chemical reactions involving not only the synthesis of cellular components but also those by which energy is provided for such synthesis. Furthermore, the term growth has only a general meaning and unless more rigidly defined contributes but little to our understanding of the role played by the endocrine glands in its occurrence and continuance.

When true growth occurs the cells of an individual organ or those of a larger part of the animal increase not only in size but also in number. This means that not only is the rate of accumulation of the characteristic cellular constituents (mainly protein, salts, and water) increased but also their absolute quantities in the organism. This continues until the animal has reached adulthood, at which time the quantity of certain constituents of the body, notably protein and inorganic salts, remains constant although the animal may continue to increase in size and weight by the addition of fat. It is important to remember that, although the quantity of protein remains constant, this constituent of protoplasm is now known to be undergoing constant breakdown and resynthesis. This knowledge has recently been gained by the brilliant work of Schoenheimer and his colleagues, who made use of the heavy isotope of nitrogen, and this information is of the utmost importance not only to our understanding of protein metabolism but also because it indicates that those processes by which protein is formed during the period of rapid growth

do not cease at adulthood but continue with the addition of some form of regulation so that the total quantity of protein in the organism is maintained within fairly narrow limits. This regulation might be accomplished by the recession of some agent that normally stimulates the anabolism of protein or by the increased activity of some factor that increases tissue catabolism, the result in either case being the establishment of a steady state.

It is now well known that the composition of the organism is not similar at different periods of growth. Moulton and his colleagues[1,2] have made detailed studies of the composition of the gain in weight of animals at different periods, and their findings are summarized. Intra-uterine growth is characterized by a relatively high proportion of water (90 per cent), a relatively low content of protein (5–9 per cent) and ash (1–2 per cent), and the deposition of fat is quite small (1–2 per cent). After birth the proportion of water in the material comprising the gain in weight decreases quite rapidly while that of protein increases to some 15 to 20 per cent. As the age of the animals advances, increasing quantities of fat are deposited and since fat is laid down with comparatively small quantities of water, the water content of the animal declines still more. When true growth ceases protein and ash are no longer accumulated but some animals, including man, may continue to increase in size and weight by further addition of fat.

Since the continued addition of fat dilutes as it were the true composition of growth, Moulton[1] has calculated for several species both the composition of the whole animals and the composition of the gain in weight on a fat-free basis. This method leads to some interesting conclusions for it is found that the proportions of protein and ash increase steadily during intra-uterine life and during the early period of extra-uterine existence. Following this, a period is reached in which the proportions of protein, ash and water remain remarkably constant, although as pointed out above very large quantities of fat may be accumulated. It is this period that Moulton has termed the "age of chemical maturity."

The interest in the steady state of the composition of the body of adult animals has been increased by the fact that the turnover of many of the elements, particularly the proteins, is continuous throughout life and consequently it may be assumed that rather exact regulatory mechanisms operate during the adult period. Among these are undoubtedly certain endocrine glands and in particular the anterior pituitary.

If it be allowed that the chemical characteristics of true growth are the

[1] Moulton, C. R.　Jour. Biol. Chem. **57**: 79.　1923.
[2] Armsby, H. P., & Moulton, C. R.　"The Animal as a Converter of Matter and Energy."　Am. Chem. Soc. Monograph Series, No. 28.　The Chemical Catalog Co.　New York.　1925.

accumulation of inorganic elements, proteins and water.[3] we are enabled by studies of the water, ash and protein metabolism of the organism to analyze the influence of any agent that either enhances or inhibits growth. It may even at this point be stated that anterior pituitary extracts do not qualitatively alter the composition of true growth but merely increase its proportion in the total weight gain.

THE ANTERIOR PITUITARY AND GROWTH

It will be agreed that the anterior pituitary is only one of many agents that determine the ultimate size and form of the whole animal and its component organs. Furthermore, there is good reason to believe that the presence of a pituitary factor is not always essential to growth. This is certainly true during the early stages of embryonic existence[4] and though the importance of the pituitary factor increases steadily in the later stages, it is now known that hypophysectomy of animals soon after birth does not lead to an immediate cessation of growth. This is particularly true in the rat where the effects of this operation have been most extensively studied. But if hypophysectomy is performed when the rats have reached a weight of 80-100 gm., growth ceases immediately even though the animal is supplied with a diet that is entirely adequate for normal growth of intact animals.

These facts are well known but the picture is not so simple as it appears at first sight. In the first place, removal of the hypophysis also removes certain other hormones whose presence is essential for the normal functions of such endocrine glands as the thyroid, adrenal cortex and gonads. These not only undergo anatomical regression but also it can be demonstrated that their function is very much reduced although not entirely obliterated. As is well known, normal growth is not possible in the absence of sufficient quantities of either the thyroid or adrenal cortical hormones and the question might well be asked, whether the cessation of growth after hypophysectomy is not due partly to the multiple glandular deficiency that follows this procedure.

In addition, hypophysectomized animals have poor appetites and consume much less food than normal animals. Whether this is a cause of growth retardation or merely a consequence of the profound changes in metabolism has not been entirely answered, but in all probability it

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[3] It is of course apparent that many other organic substances are accumulated as the organism grows, but the proteins by virtue of the unique position they occupy as constituents of protoplasm are perhaps the most important for studies of this kind. It should also be noted that the entire exclusion of lipids from the composition of true growth is also not justified since many of them are essential constituents of cells. Nevertheless, the available data suggest that the essential lipid components form only a small proportion of the gain of rapidly growing animals and that their exclusion does not greatly alter conclusions regarding the composition of the material laid down in these periods.

[4] Fugo, N. W. Jour. Exp. Zool. **85**: 271. 1940.

should be regarded as an adjustment of the organism to its new metabolic environment. It should also be recalled that removal of the hypophysis does not prevent regeneration of certain organs such as the liver. This has been clearly shown by Franseen, Brues and Richards[5] and Higgins and Ingle.[6] Both studies agree, however, that there is some retardation of the rate of regeneration which may in part be due to the smaller food consumption of hypophysectomized rats.

The most convincing evidence of the essential character of an anterior pituitary factor for normal growth is furnished by the effect of injections of extracts of this gland into hypophysectomized and normal animals. In the former case, growth is immediately resumed; in the latter, accelerated growth rates occur in young animals and those that had attained a stationary state begin to grow again. But even in these animals the part played by stimulation of other endocrine glands in the total response to the extract still needs to be taken into consideration.

It is necessary to take up in some detail the nature of the response to growth-promoting extracts in order that not only the essential action of the growth hormone may be understood but also the relation of this to the other metabolic effects of the anterior pituitary.

THE MODE OF ACTION OF THE GROWTH HORMONE OF THE ANTERIOR PITUITARY
General Effects of Growth-Promoting Extracts

Although the first association of the anterior pituitary with growth promotion was noted in individuals suffering from pituitary tumors, the first experimental demonstration that the growth rate of mammals could be accelerated by the injection of anterior pituitary extracts was carried out by Evans and Long,[7] who showed in 1921 that true gigantism could be produced in rats by this means. Later it was shown, particularly by Teel and his collaborators,[8] as well as by Evans, et al,[9] that injections of similar extracts into dogs produced not only an increased growth rate but also a condition strikingly similar to acromegaly in man.

Since most work has been and now is being carried out on rats, it may be well to outline the course of events in this species following injection with anterior pituitary extract (A.P.E.). Female rats that have reached a nearly constant weight (plateaued) are usually used. Males also respond but are less sensitive than the females. Growth is accelerated at once and in the first experiments reported the treated animals ultimately

[5] Franseen, C. C., Brues, A. M., & Richards, R. L.   Endocrinology 23: 292. 1938.
[6] Higgins, G. M., & Ingle, D. J.   Anat. Rec. 73: 95. 1939.
[7] Evans, H. M., & Long, J. A.   Anat. Rec. 21: 62. 1921.
[8] Putnam, T. J., Benedict, E. B., & Teel, H. M.   Arch. Surg. 18: 1708. 1929.
[9] Evans, H. M., et al.   Mem. Univ. California, 11. 1928.

reached a weight almost double that of their litter sisters. This growth was symmetrical, that is, the increase in length of the animal was proportional to the increased weight. While not much data have been collected on this point, such as are available are presented in TABLE 1 and are contrasted with the unsymmetrical "growth" found in rats after hypothalamic puncture. It will be observed that the A.P.E.-treated animals have ratios of body weight to body length that are somewhat greater than those found in untreated animals of the same length, a finding which indicates that the animals may have been slightly obese. As will be seen, however, there is ample evidence that true growth was induced by this treatment.

TABLE 1

WEIGHT-LENGTH RELATIONSHIPS OF:
(a) RATS TREATED WITH GROWTH HORMONE AND
(b) OBESE RATS FOLLOWING HYPOTHALAMIC PUNCTURE[10]

	Weight	Length	$\frac{B.W.}{B.L.}$ ratio
	grams	*norms*	
Normal males	372	236	1.58
" "	448	250	1.79
Males injected with A.P.E.	485	255	1.90
Normal females	220	204	1.08
" "	350	230	1.52
Females injected with A.P.E.	397	232	1.71
Normal females	300	216	1.39
Obese female litter mates	403	207	1.95

* [10] Data compiled from: Donaldson, H. H. "The Rat." Mem. Wistar Inst., Philadelphia. No. 6, 1924; Evans, H. M., & Simpson, M. E. Am. Jour. Physiol. 98: 511. 1931; Tepperman, J., Brobeck, J. R., & Long, C. N. H. Unpublished data.

At this point it is necessary to call attention to an anomalous situation. In the first experiments reported by Evans and Long the rats were injected daily for as long as 8 to 13 months with a crude alkaline extract and although growth was not as rapid in the late period as at first, nevertheless it was continuous throughout the period of injections. Later attempts to repeat this experiment, even in the same laboratory, showed that, after an initial period of brisk growth, the animals became refractory to the extract and even lost some of the weight they had gained. The same result was also obtained in hypophysectomized animals but even more discouraging was the fact that partial purification of the extract did not correct this decreased responsiveness. The development of a refractory state to anterior pituitary extracts is not limited to their growth-promoting properties and has been shown to be true for the stim-

ulation of thyroid and gonads that follows their use.   This whole subject
has been developed by Collip and others in an interesting manner and
Collip has coined the term, "anti-hormones," to describe the substances
that appear in the serum of such animals.   The reader is referred to a
recent review by Thompson[11] for a full description of present views on
this subject.

In hypophysectomized animals there may be additional reasons for
the ultimate failure to respond to growth-promoting extracts.   Evans
and his colleagues[12] have shown that many hypophysectomized animals
will again become responsive if crude extracts are substituted for the
more purified preparations or if they receive glucose injections.   It should
also be remembered that hypophysectomized animals have atroph-
ic adrenals and thyroids and that, unless these keep pace with the
increased size and metabolic demand, growth may ultimately cease, since
it is hard to conceive of any animal growing to adult size when deprived
of all but minimal amounts of the hormones of these glands.   Conse-
quently, while purification and separation of the growth-promoting agent
from the adrenotropic and thyrotropic agents can be achieved, it may
be anticipated that these extracts over a long period will prove to be
inadequate to support continuous growth in hypophysectomized and
possibly in normal animals by reason of the fact that the adrenals and
thyroid do not receive a stimulation proportional to that induced by the
growth principle in the somatic tissues.   This, however, does not ex-
clude the fact that such a preparation may induce an immediate but
temporary increase in growth.

In a recent paper Marx and coworkers[13] have studied the growth re-
sponse of hypophysectomized rats to the injection of a growth hormone
preparation that contained only small quantities of the other anterior
pituitary hormones.   The treated animals were pair-fed with a control
group.   While their body weight increased 25 per cent it was also found
that this was attended by an increase in weight of different degrees in
various organs, including the ovaries, adrenals and thyroids.   The in-
crease in the endocrine glands is particularly interesting inasmuch as the
preparation injected apparently contained only minute amounts of tropic
hormones.   This raises the question as to whether a tropic hormone is
not more concerned with the formation and liberation of specific hor-
mones than to the actual size of the endocrine glands that produce them.
As mentioned above, the role played by the quantity and quality of the

11 Thompson, K. W.   Physiol. Rev. 21: 588.   1941.
12 Evans, H. M., Pencharz, R. I., & Simpson, M. E.   Endocrinology 19: 509.   1935.
13 Marx, W., Simpson, M. E., Reinhardt, W. O., & Evans, H. M.   Am. Jour. Physiol. 135: 614.
1942.

diet in determining the response to either hypophysectomy or the injection of A.P.E. has not yet been entirely worked out. Bryan and Gaiser[14] have pointed out that the degree of response of rats is conditioned in part by the diet fed and even without growth hormone rats may, by improvements in the diet, be induced to grow to a size similar to that achieved by A.P.E. on ordinary diets.[15] Lee and Schaffer[16] have clearly shown that on a pair-fed regime, however, rats injected with A.P.E. grow more than do untreated animals. This has also been shown to be true for pair-fed hypophysectomized rats.[13]

Effects on Special Organs and Tissues

The fact that hyperactivity of the pituitary gland is associated with marked changes in certain organs and tissues of the body has been known for some time. The bony deformities, splanchnomegaly and enlarged tongue of individuals suffering from acromegaly are well known stigmata of this disease. Putnam, Benedict and Teel[8] found in a dog in which a condition resembling acromegaly was produced by chronic injection of A.P.E., that the liver was enlarged more than any other organ except the gonads and thyroid, for which organs the extract contained specific principles. The liver showed evidence of a central necrosis. Downs[17] has also noted the disproportionate increase in the liver of treated mice. The cell nuclei were greatly increased with a slight increase in cell size. There was also a marked central necrosis. In dogs there was also a marked increase in the size of the liver and central necrosis.

In experiments conducted over a short period (1–16 days) in pair-fed rats Lee and Freeman[13] have found that, although the average liver weight was significantly greater in treated animals, the number of cells per gram of tissue remained unchanged and they conclude that the liver exhibits a true hyperplasia under the influence of the extracts used and that all elements of the organ participate in it. The total quantity of nitrogen in the liver per 100 gm. of rat was 117 mg. in the controls and 129 mg. in the treated animals, indicating that in proportion to their body weight the latter had accumulated more nitrogen (protein) in their livers.

The bony changes accompanying hyperpituitarism in man have always excited interest. As is well known these are of two types: (1) a general increase in the size of the skeleton that accompanies gigantism and which occurs only when the epiphyses are open; and (2) the bony deformities

4 Bryan, A. H., & Gaiser, D. W. Am. Jour. Physiol 99: 379. 1932.
5 Anderson, W. E., & Smith, A. H. Am. Jour. Physiol. 100: 511, 1932.
6 Lee, M. O., & Schaffer, N. K. Jour. Nutrit. 7: 337. 1934.
7 Downs, W. G., Jr. Jour. Dent. Res. 10: 601. 1930.
8 Lee, M. O., & Freeman, W. Endocrinology 26: 495. 1940.

associated with acromegaly which develop when hyperpituitarism occurs during adult life.

Putnam, Benedict and Teel used bulldogs for their chronic experiments and found that the treated animals exhibited marked bony deformities. The bones were thickened and heavy and local changes such as are seen in human acromegaly were present. Evans and his colleagues[9] suggested that the choice of the bulldog for such injections may have influenced the results, as this animal may be regarded as already possessing acromegalic traits. These investigators injected a more normal type of dog (shepherd) and reported that in this breed the prolonged injection of A.P.E. did not lead to any appreciable changes in the long bones although the skull bones were enlarged. In the dachshund marked general growth was produced but the achondroplastic form of the short extremities was retained. The long bones were not particularly affected but those of the skull were thickened and enlarged. In both of these breeds the most pronounced changes occurred in the soft tissues and it is of exceeding interest that diabetes mellitus was observed to appear in two dogs after some months of treatment.

Mortimer[19] has made extensive X-ray studies not only on the changes produced by A.P.E in the skulls of normal rats but also those that follow hypophysectomy in this species. The skulls of the treated rats had less density and were more highly developed than the controls, but as Mortimer points out, the crude extracts used contain factors that stimulate the thyroid and possibly the parathyroids so that care must be used in the interpretation of the results. The changes in the skull after hypophysectomy are, however, quite characteristic. There is a marked decrease in vascularity, and the processes of bone resorption and deposition are unbalanced. All growth does not cease but the skull grows more in its transverse and vertical direction than it does in its anteroposterior diameters so that the head retains its infantile proportions and appearance. The eruption of the teeth is retarded, their outline deformed and the pulp cavities obliterated. Similar tooth changes have been described by Schour and Van Dyke,[20] and Downs[17] has reported that the injection of A.P.E. into dogs hastened the eruption of the teeth. In mice similar treatment led to a noticeable increase in the size and density of the incisor teeth and maxillae.

Histological studies on the changes in the epiphyses and related structures following hypophysectomy or treatment with A.P.E. have been

19 Mortimer, H.   Radiology 28: 5.   1937.
20 Schour, I., & Van Dyke, H. B.   Am. Jour. Anat. 50: 397.   1932.

made by several investigators. The Silberbergs[21] in particular have made extensive studies of both guinea pigs and mice. They found that extracts of beef anterior pituitary increase proliferation and may induce degenerative changes in the epiphyseal and articular cartilage and that they stimulate the deposition of bone. These effects were not prevented by thyroidectomy or ovariectomy. Freud, Levie and Kroon[22] have reported that after hypophysectomy in rats growth ceases in the tail vertebrae and that differences in the tail length can readily be detected by skiagrams as early as 7 days after operation. They also made the statement, which is somewhat surprising in view of the experience of other investigators, that the epiphyses close soon after hypophysectomy and that once completed, growth can no longer be induced by growth-promoting extracts, but if a growth-promoting extract is given immediately after hypophysectomy epiphyseal closure is prevented and tail growth continues. Not only have these investigators proposed that alterations in tail length and vertebral development be used as a method for the assay of the growth hormone but state that the growth defect after hypophysectomy is definitely localized in the epiphyseal cartilage. Consequently, they suggested that the growth hormone was a "chrondotropic" hormone, a view which was subsequently modified[23] to admit that the hormone has other sites of action. Levie and Uyldert[24] reported that removal of the adrenals does not prevent the usual effects of purified growth hormone on tail growth.

Ross and McLean[25] found that the administration of a growth preparation to plateaued rats induces histological evidences of active growth in the quiescent epiphyseal cartilage and adjacent spongiosa. They stated that this resumption of growth in the cartilage is often a better indicator of the activity of the preparation than an increase in body weight. These authors also conceded that the effect of the hormone upon the growth apparatus in the bone does not exclude the presence of specific effects in other tissues. Ray, Evans and Becks[26] have recently presented a detailed study of the alterations produced in the epiphyseal disc of rats by hypophysectomy and A.P.E.

The question of a specific pituitary factor influencing bone growth has been given a new turn by the recent observations of Ingalls and Hayes[27] that adrenalectomy is also followed by a failure of endochondrial bone

[21] Silberberg, M., & Silberberg, R.   Am. Jour. Path. 15: 547.   1939; 16: 491, 505., 1940; 17: 189.   1940.   Anat. Rec. 78: 549.   1940.   Endocrinology 29: 475.   1941.
[22] Freud, J., Levie, L. H. & Kroon, D. B.   Jour. Endocrinology 1: 56.   1939.
[23] Freud, J., & Dingemanse, E.   Acta brev. Neerland. 10: 102.   1940.
[24] Levie, L. H., & Uyldert, I. E.   Acta brev. Neerland. 9: 121.   1939.
[25] Ross, E. S., & McLean, F. C.   Endocrinology 27: 329.   1940.
[26] Ray, R. D., Evans, H. M., & Becks, H.   Am. Jour. Path. 17: 509.   1941.
[27] Ingalls, T. H., & Hayes, D. R.   Endocrinology 29: 720.   1941.

formation similar to that following hypophysectomy. These authors suggested that the atrophy of the adrenal cortex that follows hypophysectomy may be a major factor in the interruption of growth. Levie and Uyldert found, however, that adrenalectomy did not interfere with the stimulating properties of growth hormone on tail length.

It is evident, as in many other instances of the effect of the anterior pituitary on metabolism, that the changes produced in bone growth by an excess or deficiency of its hormones are exceedingly difficult to interpret, partly by reason of the number of hormones involved that may alter the processes in bones but also by reason of the fact that the long-continued injection of crude extracts results in the development of refractory states which in some instances may actually produce a hypofunction of certain endocrine glands.

Metabolic Changes Induced by Growth-Promoting Extracts

The injection of crude anterior lobe extracts into animals is followed by marked and widespread alterations in metabolism. Since such extracts contain principles other than those associated with growth promotion and since no purified growth preparation has so far been extensively tested for its effects on metabolism it is extremely difficult to interpret the large number of observations that have been reported. An attempt will be made in a later section of this paper but for the moment particular consideration will be given to the alterations in protein metabolism. The reasons for this have been outlined above and it will be sufficient to repeat that true growth is always associated with the accumulation of protein in the body and, since this can be accomplished only by an absolute or relative change in protein synthesis, the indications seem clear that either directly or indirectly the growth hormone influences these processes. The evidence for such a participation of the growth hormone is quite conclusive and indeed is strongly suggestive that the true role of the anterior pituitary in the growth process is its influence on protein metabolism. The experimental facts are as follows:

(1) The injection of anterior pituitary extract into dogs is followed by a fall in blood N.P.N., urea and amino acids of some 20–30 per cent. This occurs within a few hours of injection and may persist for some time.[28] Harrison and Long[29] have observed a fall in the blood N.P.N. of fasted rats after A.P.E. injections.

(2) Gaebler[30] has found that not only is the blood N.P.N. decreased after a single injection of A.P.E. but there is an even more striking re-

28 Teel, H. M., & Watkins, O. Am. Jour. Physiol. 89: 662. 1929.
29 Harrison, H. C., & Long, C. N. H. Endocrinology 26: 971. 1940.
30 Gaebler, O. H. Jour. Exp. Med. 57: 349. 1933.

duction in the urine nitrogen excretion. This occurs even in phlorid-zinized dogs[31] and fasted rats.[29] In Gaebler's experiments the injections also lowered the respiratory quotient and raised the basal metabolic rate. There was marked diuresis and following the injection the greater part of the retained nitrogen was excreted within the next few days.

(3) Schaffer and Lee[32] stated that the injection of A.P.E. into normal rats causes a slight fall in the urea and amino acid content of the carcass and a much greater fall in that of the liver, the urea content being reduced to less than half the initial amount. The total N.P.N. was always reduced. Prolonged treatment with A.P.E. kept the amino acid and urea content of the liver at low levels.

(4) The changes in the composition of animals whose growth has been accelerated by A.P.E. have been studied by several investigators. The first of these studies appears to be that of Downs[17] who injected mice daily for 105 days with an alkaline extract prepared by the method of Evans and Simpson. Analysis of his data indicates that the composition of the gain of the treated animals contained more water, protein and ash, and less fat than did the control animals. Wadehn[33] performed similar experiments on mice. He concluded that the excess weight gained by the treated animals could not be attributed solely to fat or water retention since the animals contained, if anything, less fat than the controls. Bierring and Nielsen[34] used rats injected with A.P.E. daily for 7 months. Analysis of the gains in weight of the treated and control groups showed that the former deposited more water and less dry matter than the latter.

In all the above experiments the animals were fed *ad libitum* and as it is now known that those treated with A.P.E. consumed greater quantities of food the suggestion of greater water and less fat retention in the treated animals assumes some significance. The best and most quantitative studies of this character are those of Lee and Schaffer[16] on rats. The control and A.P.E.-treated animals were pair-fed for a period of 77 days and the composition of the gain in weight carefully determined. Their results which are presented graphically in FIGURE 1 are compared with the composition of the gain in weight of rats becoming obese after hypothalamic puncture.[35] They show quite clearly that the injection of A.P.E. caused not only a greater total gain in weight, but what is more important is that this gain was composed largely of water and protein (83 per cent) while fat constituted only some 13 per cent. In the control

[31] Gaebler, O. H., & Zimmerman, W. J. Am. Jour. Physiol. 128: 111. 1939.
[32] Schaffer, N. K., & Lee, M. O. Jour. Biol. Chem. 108: 355. 1935.
[33] Wadehn, F. Biochem. Ztschr. 255: 188. 1932.
[34] Bierring, E., & Nielsen, E. Biochem. Jour. 26: 1015. 1932.
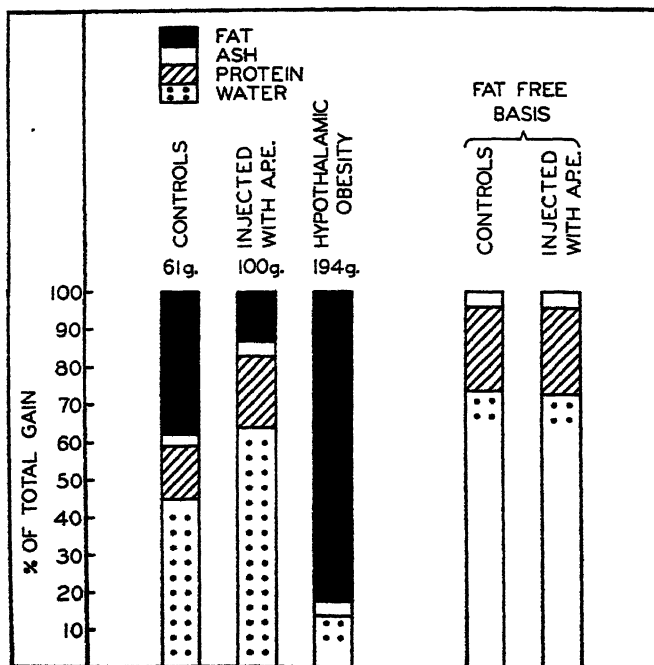[35] Hetherington, A. W., & Weil, A. Endocrinology 26: 723. 1940.

Figure 1. Composition of the gain in weight of rats after anterior pituitary treatment and hypothalamic puncture. (Data of Lee and Schaffer, Hetherington and Weil.

group water and protein made up 58 per cent of the gain while fat formed 39 per cent. In other words, under the influence of the extract the composition of the gain had reverted to that found in rapidly growing animals of a much younger age. It is also of interest to note that the extrauterine type of growth was retained and the embryonic type did not reappear, since the proportions of water, protein and ash in the gain in weight when expressed on a fat-free basis are unchanged by the treatment. This is also illustrated by a calculation of the water-nitrogen ratios as shown in TABLE 2. This ratio has a high value ranging from 60 to more than 100 in pig embryos, but after birth it falls to between 10 and 30, declining as the age of the animal increases and of course vanishing when nitrogen ceases to be retained.

The smaller increment of fat in the treated animals indicates, since the caloric intake was the same in both groups, that the treated animals had consumed a larger proportion of this substance to support their energy requirements. This stimulation of fat catabolism has been noted by all investigators to follow the injection of A.P.E. and its significance

TABLE 2

WATER-NITROGEN RATIOS OF THE GAIN IN WEIGHT AT DIFFERENT PERIODS OF GROWTH AND UNDER THE INFLUENCE OF THE GROWTH HORMONE

	H_2O	N_2	Ratio
Pig embryos			
10–15 mm. length	89.1	1.30	68.5
15–30 ” ”	90.6	1.14	80.1
30–50 ” ”	92.1	0.81	113.5
50–110 ” ”	90.9	0.93	97.6
110–160 ” ”	92.1	0.87	106.0
160–240 ” ”	87.3	1.40	62.2
Pigs after birth			
0–11 days	80.5	2.89	27.6
21–42 ”	74.9	2.56	29.2
42–96 ”	65.8	2.44	27.0
Mature pigs	22.0	1.03	21.5
Rats			
0–14 days	69.0	2.39	28.9
14–21 ”	67.0	2.27	29.5
21–42 ”	63.0	(1.48)	(42.6
42–110 ”	53.0	3.14	16.9
110–230 ”	35.8	2.59	13.8
Rats			
(Lee and Schaffer)	45.0	2.01	22.4
A.P.E.-treated controls	63.3	3.15	20.1

in relation to other metabolic activities of the anterior pituitary will be considered later.

(5) Gregory and Goss[36] found that the injection of A.P.E. into rats or rabbits causes a 50 per cent decrease in the glutathione content of the liver in 12 hours. This observation is worthy of comment although the role of this substance in metabolism is not clearly understood at present.

To summarize, it is evident that the injection of anterior pituitary extracts rich in growth-promoting activity is accompanied by marked alterations in the metabolism of proteins and related compounds. These changes indicate that under the influence of these extracts either there is an increased synthesis of protein or the normal rate of protein catabolism is reduced. It is particularly noteworthy that these effects are not limited to animals receiving food containing protein but may also be observed during fasting or in animals that have been phloridzinized. Nitrogen retention under these last circumstances is not so marked as in well fed animals but the fact that it is still present would indicate that the action of the hormone is not exactly expressed by the term "growth-promoting" but that it is in some manner intimately associated with protein metabolism.

36 Gregory, P. W., & Goss, H. Growth 3: 159. 1939.

MEDIATION OF GROWTH-PROMOTING EFFECTS
BY OTHER ENDOCRINE GLANDS

A question that always arises in considering the alterations in function produced by the injection of a pituitary extract is whether the effects in whole or in part are a result of the direct action of some constituent of the extract or whether they are a consequence of the stimulation of some other endocrine gland for which the extract employed contains a specific "tropic" agent.

In the case of such a complex phenomenon as growth it may be assumed that there are certain conditions that must be observed before this can occur. Among these are an adequate supply of foodstuffs, together with the necessary vitamins and minerals, and in addition at certain stages of development not only an adequate quantity of hormones but probably the presence of these in definite proportions to each other. It is well known that growth may be retarded or even cease altogether if certain types of foodstuffs, minerals or vitamins are lacking in the diet. It is also known that a cessation of growth may be caused not only by the removal of the pituitary but also the removal of the thyroid, adrenals or pancreas. The reason for the cessation of growth may be very different in these various instances but nevertheless it becomes necessary to examine carefully the circumstances under which a pituitary extract may influence growth and to decide if possible whether the growth-promoting action is due to some specific effect of a separate hormone secreted by the gland that acts independently of all other members of the endocrine system or whether the stimulation of growth is not in part due to the simultaneous stimulation of certain other endocrine glands. If this were the case it might well be that the liberation of several hormones from the anterior pituitary in a certain proportion to each other could be said to constitute the effective growth stimulus. The evidence that has been advanced concerning the role of other endocrine glands is summarized under the individual organs considered.

THE THYROID

Removal of the thyroid causes a virtual cessation of growth that is particularly marked if the operation is carried out at an early age. Furthermore, the injection of an excessive quantity of the thyroid hormone will also bring about a retardation or cessation of growth.

Flower and Evans[37] found that young female rats dwarfed by thyroidectomy could be made to resume growth if given A.P.E. Similar

[37] Flower, C. F., & Evans, H. M.   Anat. Rec. **29**: 383.   1924.

results were found with adult thyroidectomized female rats. Smith and his collaborators[38] found that thyroid extract would not induce growth in hypophysectomized rats but the animals responded to A.P.E.

Later, Smith[39] reported that if both the thyroid and hypophysis were removed, better growth was obtained with A.P.E. if thyroid extract was simultaneously administered. Evans, Simpson and Pencharz[40] have repeated the earlier work of Flower and Evans and though confirming their observations have also found that the response to A.P.E. was greater if the animals were also given thyroid hormone. In their experiments also thyroxin failed to promote the growth of thyroidecto-mized-hypophysectomized animals.

It should be noted that in all these experiments thyroidectomy was performed sometime after birth and evidently this is a point of importance because Salmon[41] reported that if the rats are thyroidectomized at birth there follows not only a remarkable stunting of the animals but also an absolute failure to respond with a resumption of growth to the implantation of pituitary tissue or the injection of A.P.E. She suggested that the thyroid hormone is responsible for the development of the capacity of the organism to respond to other hormones.

Added interest has been given to these studies on the relation of the thyroid to the growth-stimulating properties of the pituitary by the work of Riddle and his collaborators. This work may be said to have begun by the observation of Bates, Laanes and Riddle[42] that the administration of prolactin, desiccated thyroid, or thyrotropic hormone to dwarf mice promotes growth and that there exists a synergism between prolactin and thyrotropic hormone which is sufficient to account for the growth-promoting effects of A.P.E. At that time these authors expressed the opinion that the "growth hormone" as a separate entity did not exist. Further work along these lines by this group of investigators has been largely confined to studies of the influence of various pituitary preparations on the growth and metabolism of hypophysectomized pigeons[43] and though their results suggest that prolactin may be of especial significance in its ability to maintain the appetite and promote both body growth and that of the intestinal organs in this species there is little to suggest that it has any such effect in a mammal. Indeed, Evans[44] in a spirited reply to their claims regarding the existence of a separate growth principle has pointed out that: (a) anterior pituitary extracts practically free of pro-

8 Smith, P. E., Greenwood, C. F., & Foster, G. L. Am. Jour. Path. 3: 669. 1927.
9 Smith, P. E. Proc. Soc. Exp. Biol. & Med. 30: 1252. 1933.
0 Evans, H. M., Simpson, M. E., & Pencharz, R. I. Endocrinology 25: 175. 1939.
1 Salmon, T. N. Endocrinology, 23: 446. 1938; 29: 291. 1941.
2 Bates, R. W., Laanes, T., & Riddle, O. Proc. Soc. Exp. Biol. & Med. 33: 446. 193
3 Schooley, J. P., Riddle, O., & Bates, R. W. Am. Jour. Anat. 69: 123. 1941.
4 Evans, H. M. Proc. Assn. Res. Nerv. Mental Dis. 18: 175. 1936.

lactin and thyrotropic hormone still produce an acceleration of growth in both normal and hypophysectomized rats; and (b) the injection of purified prolactin either alone or in combination with a potent thyrotropic extract failed to do so. White, Catchpole and Long[45] have also reported that their highly purified crystalline preparation of prolactin in doses of 4 mg. a day did not induce growth in hypophysectomized rats.

Apart from those experiments in which an increase in body weight has been taken as a measure of growth-promoting activity it has been shown by Gaebler[46] that thyroidectomy does not prevent the nitrogen-retaining effect of A.P.E. in the dog.

It may be concluded that, unless thyroidectomy is performed very early in life, the absence of this endocrine gland does not prevent either the ability of anterior pituitary extracts to induce growth nor their capacity to bring about nitrogen retention. Furthermore, the thyroid hormone is unable to induce growth in hypophysectomized animals.

The Adrenal Cortex

Though the evidence concerning the relation of the thyroid to the growth-promoting action of pituitary extracts is in general in agreement, the role of the adrenal cortex has not as yet been thoroughly investigated. The reasons for this are several and include not only the severe and rapidly fatal deficiency that occurs following removal of the adrenals but also the fact that adequate replacement therapy for this deficiency is a recent development. Certain observations have been made, however, that suggest that although the growth response to A.P.E. may be restricted in animals deprived of their adrenals nevertheless it is still evident. The administration of such potent adrenal steroids as corticosterone or 11-dehydro-17-hydroxy corticosterone does not cause growth in hypophysectomized rats.[47] although it is still an open question as to whether the use of such steroids along with purified growth extracts would not result in better growth than if the pituitary extract alone were given.[48] Indeed, as mentioned above, the superiority of crude extracts over long periods of injection may well be due to their added complement of adrenotropic (and thyrotropic) hormone.

The enhancement of growth in adrenalectomized animals by pituitary implants has been studied by Emery and Gottsch.[49] They reported that female adrenalectomized rats that received pituitary implants from

[45] White, A., Catchpole, H. R., & Long, C. N. H.   Science 86: 82.   1937.
[46] Gaebler, O. H.   Am. Jour. Physiol. 110: 584.   1935.
[47] White, A., & Long, C. N. H.   Unpublished data.
[48] It should be pointed out that the administration of these steroids or of cortical extracts greatly increases the capacity of hypophysectomized rats to resist such stresses as fasting, insulin injection or exposure to cold.
[49] Emery, F. E., & Gottsch, L. G.   Endocrinology 28: 321.   1941.

castrated rats grew slightly faster than did their normal controls and far better than untreated adrenalectomized rats. Although the primary purpose of the investigation was to study the influence of *corpora lutea* formation on the survival of adrenalectomized rats this observation is of significance in indicating that pituitary implants are capable of producing better than normal growth in rats deprived of their adrenal glands. Furthermore, it should also be noted that the substitution of a potent gonadotropic extract, though effective in prolonging life, did not cause significant gains in weight.

In earlier experiments with alkaline extracts Evans and his associates[9] noted that although these preparations did not prolong the life of adrenalectomized rats they did in some instances produce a gain in weight. But this gain in weight was observed in only one group of rats that survived the operation for 20 days or more while those that lived less than this time always lost weight. Shumacker and Firor[50] have also found that pituitary implants do not influence the loss of weight or survival of adrenalectomized rats.

In our laboratory, Miss Fry and I have frequently administered growth-promoting extracts to adrenalectomized and partially depancreatized rats. In some animals no supportive treatment except the administration of 1 per cent NaCl solution as drinking fluid was given. In others the animals received a constant daily dose of adrenal cortical extract that was added to the drinking water. In general, it may be stated that even when the rats received only sodium chloride the injection of either crude or partially purified growth-promoting extracts nearly always was followed by a gain in weight. When the rats received a constant daily dose of cortical extracts, excellent gains in weight were always observed as shown in FIGURES 2 and 3. Our experience suggests that although A.P.E. is effective in causing a gain in weight in the absence of any cortical hormone nevertheless better responses always followed treatment with it. This, of course, might have been expected but the real point of these experiments is that, although the presence of some cortical hormone is necessary for a maximal response, such effects are not dependent on the presence of cortical cells.

However equivocal the influence of A.P.E. on the growth of adrenalectomized rats may appear there is another line of evidence that suggests (provided the severe results of adrenal insufficiency are avoided) that the protein metabolism of such animals responds in the same manner, if not the same degree, as normal animals to the injections of extracts that produce growth in the latter. Harrison and Long[29] injected

[50] Shumacker, H. B., Jr., & Firor, W. M. Endocrinology **15**: 676. 1934.
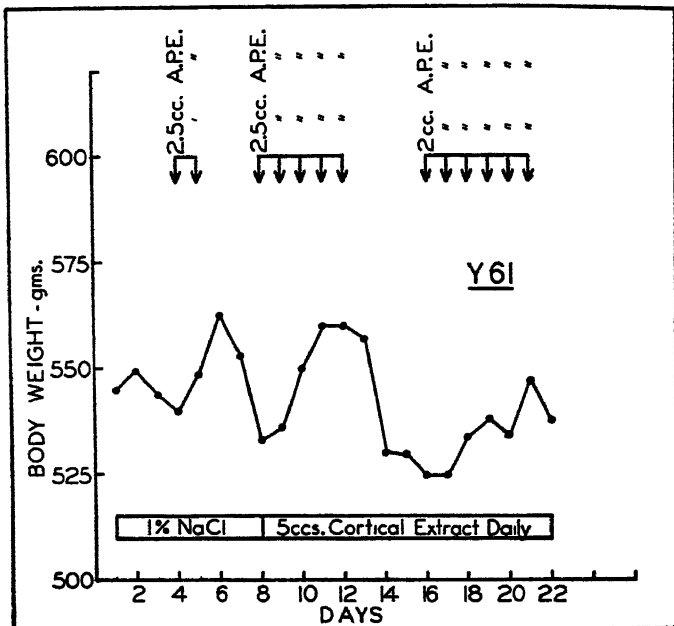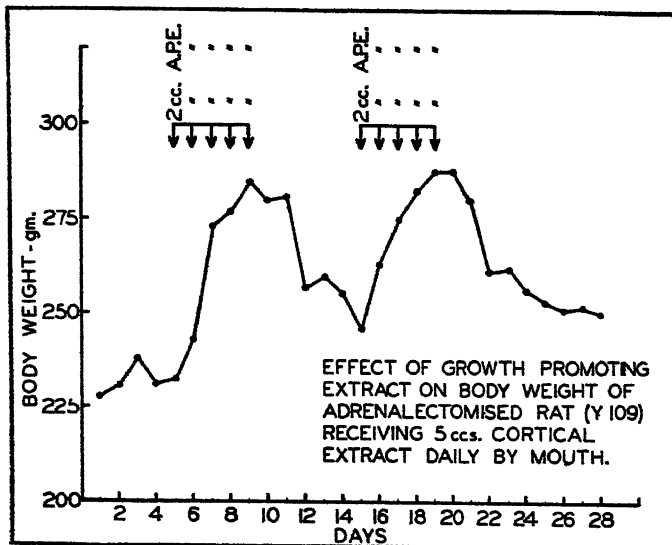
FIGURE 2.

FIGURE 3. Effect of growth-promoting extract on body weight of adrenalectomised rat (Y 109)
receiving 5 cc. cortical extract daily by mouth.

fasted adrenalectomized rats with A.P.E. and observed not only a fall in urine nitrogen of the same magnitude as found in intact animals but also a decrease in the blood N.P.N. It was also observed that the blood glucose level was decreased, particularly in the adrenalectomized rats, and that acetonuria was present after the injections.

Dr. Gaebler has permitted me to cite an experiment he has recently carried out on a dog with thyroid, pancreas and both adrenals removed. The diet was kept constant and the animal received daily injections of insulin and cortical extract. When 100–200 mg. of a potent growth-promoting preparation were injected in one dose there followed a moderate degree of nitrogen retention which, although less in magnitude than that previously found by him in normal animals, was nevertheless quite apparent. The weight of the animal also increased slightly after the injection.

The Pancreas

It has long been recognized that the injection of insulin decreases the nitrogen excretion in the urine provided ample carbohydrate is supplied in the diet. It is also common knowledge that an absent or deficient supply of insulin causes cessation or retardation of growth.

The particular interest in the role of the pancreas on the growth-promoting action of the anterior pituitary lies in the fact that the injection of crude extracts is followed by evidence of an impaired carbohydrate tolerance in most species and in some, particularly the dog, by glycosuria and ultimately a permanent diabetic state.

Since the growth-promoting and "diabetogenic" action are always closely associated in anterior pituitary extracts it is a point of considerable interest to know whether or not these two effects are different manifestations of the same hormone. This point will be considered in more detail in a later section; for the moment the question as to whether the presence of an intact pancreas is necessary for the growth-promoting action of A.P.E. may now be considered.

Mirsky[51] has suggested that the nitrogen-retaining effect of A.P.E. is due to stimulation of the islands of Langerhans by a "pancreatotropic" hormone and that the increased insulin supply not only decreases deamination in the liver but also increases nitrogen retention (protein synthesis) in the muscles. If this view is correct a depancreatized animal should show a notable failure to retain nitrogen when A.P.E. is injected. Mirsky believes he has demonstrated such a failure by his observations that A.P.E. increases the rate of accumulation of non-protein nitrogen

[51] Mirsky, I. A. Endocrinology **25: 52. 1939.**

in the blood of nephrectomized-depancreatized dogs, but the same extract given to nephrectomized but otherwise normal dogs results in a decreased rate of accumulation of these intermediary metabolites. He also supports this hypothesis by his observation that the injection of insulin also decreases the accumulation of the blood N.P.N. in normal, eviscerated and depancreatized dogs.[52]

Gaebler and Galbraith,[53] however, have carried out carefully controlled balance experiments on depancreatized dogs receiving a standard quantity of insulin and then injected with A.P.E. and conclude that an increased insulin output is not the immediate and only cause of the observed nitrogen retention although they concede that the presence of some insulin may be an essential condition for it. Finally, as mentioned above, Gaebler has shown that even if the thyroid and adrenals are removed in addition to the pancreas, nitrogen retention can still be observed after A.P.E. injection.

Since the above was written Young[54] has shown that when puppies in contrast to adult dogs are treated with A.P.E. they do not develop glycosuria, at least not until the injections have been carried on for some months; but they do respond by a marked gain in weight and nitrogen retention. Young suggests that these changes are associated with an increase in the quantity of islet tissue which can be regarded as then secreting sufficient insulin to neutralize at least for a time the "diabetogenic" activity of the extract.

In view of this and other work from his laboratory, Young suggests that the A.P.E. contains at least two principles that influence metabolism: (1) a pancreatotropic hormone which increases insulin secretion which in turn increases nitrogen retention and causes a gain in body weight; and (2) a diabetogenic hormone which either suppresses carbohydrate oxidation or increases carbohydrate formation.

Such a view might be interpreted to indicate that the pancreatotropic hormone through its influence on insulin secretion was a growth-promoting hormone, although it is hardly likely that the failure of hypophysectomized animals to grow is conditioned by an inadequate supply of insulin. It seems more reasonable to conclude that continuous growth requires a proportionately larger supply of insulin (and other hormones) and that so long as this can be maintained the growth hormone of the anterior pituitary will continue to operate in a normal manner. If the secretion of insulin becomes inadequate to meet the metabolic demands

[52] Mirsky, I. A. Am. Jour. Physiol. **124**: 569. 1938.
[53] Gaebler, O. H., & Galbraith, H. W. Endocrinology **28**: 171. 1941.
[54] Young, F. G. Brit. Med. Jour. **2**: 897. 1941.

imposed by rapid growth, the usual consequences of such a defect become manifest.

The Gonads

Although removal of the glands may influence the growth rate to some extent there is ample evidence that it does not modify in any significant manner the growth response to the injection of A.P.E. It should be remembered, however, that Kochakian and Murlin[55] and Kenyon and coworkers[56] have shown that the injection of testosterone propionate is followed by striking nitrogen retention and a gain in weight in dogs and man, respectively. In the experiments on human beings it could be calculated that a considerable part of this gain in weight was due to the deposit of newly formed protein in tissues other than those of the genital organs. Though it is improbable that such effects could be demonstrated in hypophysectomized animals nevertheless these experiments emphasize the complexity of the endocrine relationships controlling growth.

The Thymus

Although Bomskov and Sladovic[57] claimed that the diabetogenic effects of the anterior pituitary are mediated by the thymus, Reinhardt, Marx and Evans[58] have been able to show that at least as far as the growth-promoting action of A.P.E. in rats in concerned, removal of the thymus is without effect. It may be noted, however, that in a recent paper Marx and coworkers[13] found that the thymus gland of hypophysectomized rats treated with a purified growth preparation grows to a greater degree than that of any other organ examined.

It can be concluded that, although the absence of such essential hormones as these of the thyroid, adrenal cortex, or pancreas may obliterate or greatly modify the usual growth-promoting or nitrogen-retaining properties of anterior pituitary extract, there is little evidence to suggest that either of these effects is mediated by these endocrine glands and consequently it would appear that there exists in the anterior pituitary a specific agent that directly affects the tissues and whose action results in an increased retention of protein and an acceleration of the growth rate.

[55] Kochakian, C. D., & Murlin, J. R.   Jour. Nutrit. 10: 437.   1935; Am. Jour. Physiol. 117: 642.   1936: Endocrinology 21: 750.   1937.
[56] Kenyon, A. T., Knowlton, K., Sandiford, I., Koch, F. C., & Lotwin, G.   Endocrinology 26: 26.   1940.
[57] Bomskov, C., & Sladovic, L.   Pfluger's Arch. ges. Physiol. 243: 611.   1940.
[58] Reinhardt, W. O., Marx, W., & Evans, H. M.   Proc. Soc. Exp. Biol. & Med. 46: 411.   1941.

IS THE GROWTH HORMONE A REGULATOR OF
PROTEIN METABOLISM?

It has been stressed that not only is true growth characterized by an increased rate of protein synthesis but also that the material laid down in the gain in weight has a rather constant composition. It has also been noted that many other procedures besides hypophysectomy may inhibit growth, but the specific effect of the anterior pituitary appears to be its ability either to accelerate the growth rate of a growing animal or to cause a striking resumption of growth in those species in which it has practically ceased and in which certain prerequisites such as open epiphyses are still present. If a resumption of skeletal growth is not possible because of epiphyseal closure, it has been shown that this agent will cause a resumption of growth in the soft tissues of the body.

In approaching the question as to whether the growth hormone produces these effects solely by virtue of its influence on certain phases of protein metabolism, it is well to recall:

A. The fall in the urea and amino acid nitrogen content of the blood and liver begins soon after the injection of A.P.E. and persists throughout its period of action. This is also true of the decrease in the glutathione content of the liver and muscles observed by Gregory and Goss.

B. Nitrogen retention and a decrease in the non-protein nitrogenous constituents of the blood can still be produced in fasting or phloridzinized animals. Under such circumstances growth is of course not possible; indeed the animals are losing weight.

C. Most of the weight gained by animals after short periods of treatment with A.P.E. is lost when treatment is stopped, but if treatment is continued progressively smaller parts of the weight increment are lost when A.P.E. is finally withdrawn.[59] This strongly suggests that under the action of this hormone protein or its derivatives are first accumulated in a highly labile form, but as time goes on more and more of the retained protein is converted into forms which are not so easily yielded again.

D. The important role of the liver in protein metabolism has naturally directed attention to this organ as a possible site of the first action of the hormone.[59] It has been observed that the liver increases in size out of proportion to the rest of the body in animals treated with crude growth-promoting extracts.[60] Furthermore, the immediate changes

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[59] **Lee, M. O.** Proc. Assn. Res. Nerv. Mental Dis. **18**: 193. 1936.

[60] Doubt on the relation of the growth hormone to the increase in liver size is raised by a recent paper by Fraenkel-Conrat, Simpson, & Evans.[61] These investigators found that purified growth hormone causes only a slight absolute increase and a significant relative decrease in the liver weight of hypophysectomized rats, whereas purified thyrotropic hormone or thyroxin bring about both a relative and absolute increase in liver weight.

[61] **Fraenkel-Conrat, H. L., Simpson, M. E., & Evans, H. M.** Am. Jour. Physiol. **135**: 398. 1942.

in such nitrogenous constituents of tissues as amino acids, urea and glutathione are far more striking in the liver than the rest of the body. It is also known, particularly from the work of Addis, Poo and Lew[62] that in a 2-day fast rats lose 20 per cent of their protein but only 4 per cent from the rest of the body. We have frequently observed that rats when hypophysectomized and then immediately fasted for 2 days excrete as much as 50 per cent more nitrogen than intact rats.

E. The work of Mirsky[51] indicates that nitrogen retention does not follow A.P.E. injection into eviscerated (liverless) animals and some preliminary experiments by Miss Frame in this laboratory also suggest that the usual decrease in blood amino acids is not elicited by A.P.E. in eviscerated rats

Apart from the question as to whether the sole primary site of action of the growth hormone is in the liver, a fairly reasonable hypothesis can be advanced that the participation of this agent in the growth process is due to its influence on protein metabolism. The nature of this effect is quite unknown but in general terms it is expressed either by an increased rate of formation of proteins or their immediate derivatives or by an inhibition of the rate of protein catabolism.

There are certain other implications of this statement that are of interest. The hypophysectomized animal not only has a disordered metabolism that is a consequence of the absence of the growth hormone but also exhibits the characteristic changes that follow hypofunction of other endocrine glands. The injection of a growth preparation freed from the tropic hormones may be expected to cause nitrogen retention and initiate growth but it is still a very open question as to whether such growth could be long continued if such organs as the adrenal cortex and thyroid were unable to increase their output of hormone to meet the increased demands placed on the organism by a continued increase in size. Questions such as this lead one to wonder whether the use of the term "growth" hormone for this pituitary agent does not place too great an emphasis on this particular agent as the only hormonal factor that is necessary for growth to occur. There is no doubt of its importance and probably little doubt that it is a separate entity but nevertheless as our knowledge of its sphere of influence is more fully defined it seems entirely probable that some other designation will ultimately be assigned to it than the one used at the present time.

When most animals reach a certain age growth practically ceases. Is it to be assumed that under these conditions the growth-promoting activity of the anterior pituitary is in abeyance? There is good evidence

62 Addis, T., Poo, L. J., & Lew, W. Jour. Biol. Chem. 115: 117. 1936.

that the growth hormone content of the gland is not greatly different in adult animals from that found in young animals at their most rapid period of growth. The answer to this may lie in the fact that protein metabolism is never in a stationary state and that the difference between young and adult animals is that in young animals protein synthesis is greater than protein breakdown, but in adult animals a fairly exact balance has been struck between the two. The work of Schoenheimer and Rittenberg[63] has clearly indicated that a constant exchange is going on between the nitrogen derived from the diet and that of the tissues and that, far from being the "building blocks" of living cells, the protein molecules are continually undergoing transformation. Furthermore, the rather exact balance in protein metabolism maintained in adult animals suggests the existence of factors capable of regulating both the anabolic and catabolic phases.

The evidence outlined above indicates that the growth hormone of the anterior pituitary is one of these regulatory factors and that its operation displaces the metabolism so that protein retention occurs. Such a displacement operates during the period of growth but in later life either its influence is less intense or other factors that accelerate protein catabolism come into greater prominence. Indeed, there is already some evidence that the secretions of the adrenal cortex influence protein metabolism in an opposite manner to that of the pituitary growth factor. Since, however, the functional activity of the adrenal cortex itself is controlled by the anterior pituitary this organ can adjust the protein metabolism within a wide range.

In spite of these generalizations it must be frankly admitted that little is really known of the mechanism of action of the growth hormone and to couple it with the problem of protein synthesis merely transfers the question to another field in which only fragmentary knowledge is available. Nevertheless, the continued study of this unique hormone may ultimately prove to be exceedingly valuable in this most difficult field of biochemistry.

In conclusion, it should be noted that Paschkis[64] and Paschkis and Schwoner[65] suggest that the factor which produces a decrease in the non-protein nitrogen of the blood is not identical with that which promotes growth. The main point of differentiation appears to be that gelatine feeding still produces a fall in the N.P.N. content of the blood of pituitary dwarfs who, of course, do not grow. In my opinion stronger evidence than this will be required to establish the separate identity of the

[63] Schoenheimer, R., & Rittenberg, D.   Physiol. Rev. **20**: 218.   1940.
[64] Paschkis, K. E.   Endocrinology **23**: 368.   1938.
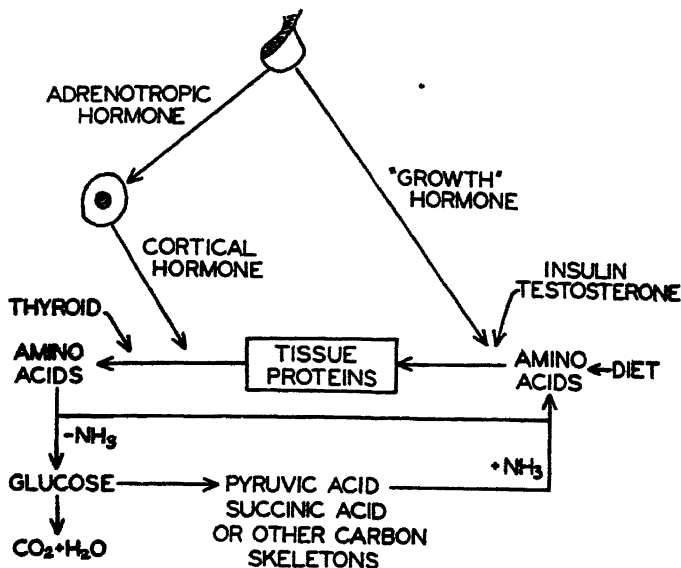[65] Paschkis, K. E., & Schwoner, A.   Endocrinology **26**: 117.   1940.

ENDOCRINE CONTROL OF PROTEIN METABOLISM

FIGURE 4. Endocrine control of protein metabolism.

two hormones.   In FIGURE 4 is shown the endocrine factors that appear to regulate protein metabolism and their possible points of action.

ASSAY OF THE GROWTH HORMONE

The two universally used methods for the assay of the growth hormone are (a) the gain in weight produced in plateaued female rats and (b) the resumption of growth and consequent gain in weight of hypophysectomized rats.   Both methods are laborious and time-consuming, particularly the latter, but at present they are the only methods that permit a reasonable degree of accuracy.

Evans and his colleagues[66] have undoubtedly had the most extensive experience in the assay of growth-promoting extracts.   In their work they have used plateaued female rats in groups of 6 or more animals and judged the response by the average weight gained over a period of 20 days with 17 injections.   The unit of activity was defined as the amount of material producing an average gain of 40 gm. in body weight per rat in this period.   Their data show a linear response between growth and dose over a twentyfold increase in the material administered.   Other

[66] Marx, W., Simpson, M. E., & Evans, H. M.   Endocrinology 30: 1.   1942.

investigators have used shorter periods of injection, some as short as 3 days, but as Evans points out the slope of the dose-response curve increases up to 15 days and consequently a longer period insures greater accuracy. Also in our laboratory we have frequently observed that temporary gains in weight (water retention ?) are not uncommon after the injection of the cruder extracts which probably contain appreciable amounts of posterior lobe hormones and possibly others such as the adrenotropic hormone which may also influence the electrolyte and water balance.

Many investigators have preferred to use the hypophysectomized rat as a test object. To supply and maintain sufficient numbers of these animals is no easy matter, although for "spot" tests of growth-promoting activity they can be advantageously used since a weight increase can usually be detected after only a few injections of an active preparation. They are also probably 10 times as sensitive as intact animals. Van Dyke and Wallen-Lawrence[67] have devised a method of assay in which only 3 daily injections are given. Although this method does not yield good quantitative results, it is undoubtedly useful for following the growth-promoting activity when fractionation of pituitary extracts is being carried out.

The majority of those who have used hypophysectomized rats for assay purposes have carried the injections over longer periods (7–20 days) and most workers have defined their unit of activity as the quantity of material that will produce an average gain of 1 gm. a day for the period chosen. It goes without saying that in carrying out these tests the ordinary precautions regarding biological assay must be observed. Among these are: the use of animals of a uniform age and strain who have been raised under identical conditions of diet and environment; the use of a sufficient number of animals at each dose level tested and the use of doses of the active principle that are submaximal in their effects; finally, although this is perhaps the most difficult to meet at the present time, in the case of the growth hormone the simultaneous injection of comparable groups of animals with a standard preparation of the material under test.

Observing such precautions Bülbring[68] has been able to show that for an injection period of 7 days there is a linear relationship between the gain in weight and the logarithm of the dose when 6 hypophysectomized rats are used in each group. She further notes that the response is not so uniform if the animals are used more than once.

⁶⁷ Van Dyke, H. B., & Wallen-Lawrence, Z. Jour. Pharmacol. & Exp. Therap. 40: 413. 1930.
⁶⁸ Bülbring, E. Quart. J. Pharm. & Pharmacol. 11: 26. 1938.

In the past there has been some discussion regarding the relative merits of the plateaued female rat and the hypophysectomized rat as test objects for growth-promoting activity[69] and, although there is much to be said in favor of the latter, the recent studies of Evans and his colleagues have shown that normal rats can be used as successfully as hypophysectomized ones. They also have the added advantage of possessing an intact endocrine system, a fact which as Evans states may be of importance as the purification of the growth-promoting activity is achieved.

Freud, Levie and Kroon[22] and Freud and Levie[70] have advocated a different method of assay that is based on their observation that growth of the tail vertebrae ceases after hypophysectomy but can be made to resume by injecting growth hormone. They indicate that treatment must be started soon after operation, otherwise the epiphyses will be closed and no response can then be obtained. They advocate the measurement of the tail length, preferably in skiagrams, as an index of growth promotion. Probably owing to the war no extensive quantitative data have been available for study, but Evans points out that in the experience of his laboratory tail growth continues for some time after hypophysectomy in young rats and that in any case measurement of tail length is no more accurate, if as accurate, as measurement of body weight. The further claim of Freud and his collaborators that the specific effect of growth hormone is a chondrotropic one is commented on elsewhere.

Attempts have been made to use certain alterations in the constituents of blood and tissues as a measure of growth-promoting activity; among these is the decrease in glutathione content of the liver after A.P.E. injection. Lee[59] commented that he found this method rather impractical and also stated that similar results followed an attempt to use the decrease of urea, amino acid, nitrogen, etc., in the tissues. But if it is ultimately shown that the same agent is responsible both for growth stimulation and the alterations in nitrogen metabolism, it would seem worthwhile to investigate more thoroughly the question as to whether some phase of protein metabolism might not be adopted as an assay method since there would be a considerable saving of time by the use of metabolic changes extending over a period of hours instead of days.

[69] Chou, C., Chang, C., Chen, G., & Van Dyke, H. B.   Endocrinology **22**: 322.   1938.
[70] Freud, J., & Levie, J.   Arch. internat. pharmacodyn. et therap. **59**: 232.   1938.

PURIFICATION OF THE GROWTH HORMONE

The problem of the isolation of the growth hormone of the anterior pituitary is of interest not only because the preparation of the hormone in such a form would permit its chemical characteristics to be determined but also because it would then be possible to determine how many other metabolic activities were associated with the gland.

Although at the present time this desirable goal has yet to be achieved considerable progress has recently been made, so that now certain general statements can be made concerning its chemical characteristics. The substance is either a protein or a closely related protein derivative. It is destroyed by heat, inactivated by proteolytic enzymes and precipitated by the usual protein precipitants. It possesses a limited pH stability, being inactivated by strong acid solutions but is more stable in alkaline solutions. Consequently, the methods employed to extract the hormone from the gland have usually begun with the use of mildly alkaline or neutral solutions. Evans and his colleagues have employed $Ba(OH)_2$ or $Ca(OH)_2$ since these metals are easily removed. Others have used NaOH and Bonsnes and White[71] used 2 per cent NaCl solutions. Such initial extracts contain not only a major part of all the pituitary hormones but also considerable quantities of inert proteins. With such extracts a large part of the work on the phenomenon of growth promotion and other phases of anterior pituitary physiology has been done, but it is well to remember the essential crudity of composition of these extracts.

Further purification of the growth (and other principles) has followed the usual methods employed for the fractionation of protein mixtures: (a) precipitation with Na_2SO_4 or $(NH_4)_2SO_4$ and (b) isoelectric precipitation.

Thus Van Dyke and Wallen-Lawrence[67] adjusted the alkaline extract to pH 7.2 and added Na_2SO_4 to make the final concentration 20 per cent. The precipitate contained all the growth-promoting activity. The Na_2SO_4 precipitate was then dialyzed free from inorganic salt and the resulting solution adjusted to pH 4.8, the precipitate discarded and the supernatant was used both for animal and clinical studies. Bugbee, Simond and Grimes[72] also used the globulin fraction precipitated by Na_2SO_4 as their working solution. Extracts of this type, however, undoubtedly contain other pituitary hormones.

Evans and his colleagues have published a number of papers during the last 10 years on the purification of this hormone. In their first paper[9] there are extensive data on comparative methods of extraction and some

[71] Bonsnes, R. W., & White, A. Endocrinology **26**: 990. 1940.
[72] Bugbee, E. P., Simond, A. E., & Grimes, H. M. Endocrinology **15**: 41. 1931.

of the properties of the hormone. In 1938 they reported[73] the use of $(NH_4)_2SO_4$ as a precipitating agent for the hormone. The alkaline extract was progressively precipitated by increasing the $(NH_4)_2SO_4$ concentration in steps from $\frac{1}{5}$ to $\frac{1}{2}$ saturation. The lower concentration did not precipitate the growth activity but half saturation did. This precipitate was freed from a good deal of inert material by repeated re-solution and precipitation but the final 'L' precipitate still contained considerable quantities of lactogenic, thyrotropic and gonadotropic hormones in addition to the growth hormone. The relative distribution of the 4 hormones had, however, been considerably altered from that found in the original extract.

From this 'L' fraction considerable quantities of gonadotropic hormone could be removed by extraction with 5–10 per cent NaCl in which it is much more soluble than the growth hormone. To remove the larger part of the lactogenic hormone either extraction with 0.1 saturated $(NH_4)_2SO_4$, in which it is more soluble than the growth hormone, or treatment with bromine water was employed. The last procedure precipitates the lactogenic hormone, and the growth principle can be recovered by saturating the supernatant with NaCl. Efforts to remove all the thyrotropic hormone were not too successful but a few preparations were obtained that contained only small quantities of this activity.

The whole procedure gave about a 70 per cent yield of growth hormone but the increase in potency of only fivefold was somewhat disappointing since so much of the other hormones had been removed.

In their most recently published paper[74] these investigators have introduced treatment of the "globulin" fraction precipitated by $(NH_4)_2SO_4$ with cysteine as a method of removing other hormones. To employ this, concentrated protein solutions (5–7 per cent) are treated with alkaline cysteine solution at room temperature for 1–2 days. The precipitate that forms is removed and the growth hormone isolated from the supernatant. This preparation contained 15.7–16.3 per cent nitrogen and practically no glucosamine in contrast to the gonadotropic hormone. It had about twice the growth activity of the 'L' fraction but still possessed considerable adrenotropic activity. It is of particular interest that this preparation lowered the respiratory quotient of glucose-fed rats and also stimulated ketogenesis.

Both these procedures, though producing some increase in potency, appear to be directed more toward removal or inactivation of other hormones than actually effecting an isolation of the growth principle.

[73] Evans, H. M., Uyei, N., Bartz, Q. R., & Simpson, M. E. Endocrinology **22**: 483. 1938.
[74] Fraenkel-Conrat, H. L., Meamber, D. L., Simpson, M. E., & Evans, H. M. Endocrinology **27**: 605. 1940.

Collip, Selye and Thomson[75] extracted the glands with 1 per cent NaOH or NH₄OH. The mixture was acidified with acetic acid and filtered; the residue was re-extracted with alkali and the acidification was repeated. The combined supernatants were made alkaline with 1 per cent NH₄OH and sufficient quantities of calcium chloride and sodium phosphate were added to form a suspension of calcium phosphate. Ammonia was removed by vacuum distillation and the calcium phosphate was collected and extracted with 0.5 per cent NaOH. This alkaline extract was adjusted to pH 6.5, made alkaline with NH₄OH and concentrated *in vacuo*. A precipitate settled out at pH 7.5–8.0 which was removed and extracted with alkali. This solution was neutralized and proved to have marked growth-stimulating properties. Such extracts were free of gonadotropic and thyrotropic hormone but contained prolactin.

Dingemanse[76] and Dingemanse and Freud[77] have used somewhat different procedures to produce an active growth preparation which they claimed is free from lactogenic and thyrotropic hormones. The material is absorbed on "Norite" from alkaline extract of acetone-dried glands. The hormone was elicited from the "Norite" with liquid phenol. This solution was poured into alcohol-ether and the precipitate collected. Empirical analysis showed a typically protein composition. They stated that the hormone is destroyed by heat, strong acids and alkalis, and is inactivated by proteolytic enzymes. They also stated that the active principle is ultrafilterable through collodion membranes of 30μ porosity. No lactogenic, thyrotropic, gonadotropic or adrenotropic activity could be detected at 30γ levels which was about 3 times the quantity required to produce a gain of 1 gm. a day in hypophysectomized rats.

In the earlier paper[77] these investigators also stated that quite an active growth preparation can be obtained by simply dialyzing a weak alkaline extract through collodion. After treatment of the dialysate with "Norite," active preparation was obtained that contained only 4.84 per cent (?) of nitrogen.

It should be noted that the claims for such a high degree of potency of this preparation could not be substantiated by Evans[44] and that Schooley, Riddle and Bates[43] stated that a preparation obtained from the Amsterdam laboratory contained detectable quantities of prolactin and traces of thyrotropic and gonadotropic activity and that in a daily dose

[75] Collip, J. B., Selye, H., & Thomson, D. L.   Proc. Soc. Exp. Biol. & Med. 30: 544.   1933.
[76] Dingemanse, E.   Kongressbericht des XVI Internationalen Physiologen Kongress.   Frei Vereinigung Schweiz. Physiologen. Zurich.   1938.   p. 320.
[77] Dingemanse, E., & Freud, J.   Acta brev. Neerland. 5: 109.   1935.

of 0.1 mg. it did not stimulate growth in hypophysectomized pigeons which was a marked contrast to the effect of 0.067 mg. of prolactin.

Fevold and coworkers[78] have reported a method by which 5 anterior lobe hormones can be separated into 5 different protein fractions. When precipitation of a pH 8 extract was carried out at pH 5.4 in the presence of 0.25 M $(NH_4)_2SO_4$ the growth, lactogenic, thyrotropic and gonadotropic hormones remained in the solution. The growth hormone was then precipitated by addition of $(NH_4)_2SO_4$ to 1.8 M concentration. Teel[79] had previously reported precipitation of the growth activity by 1.4 M Na_2SO_4. The precipitate obtained, after removal of $(NH_4)_2SO_4$, was extracted with 0.25 M $(NH_4)_2SO_4$ at pH 7.0. The solution was adjusted to pH 5.4 and an inert precipitate removed, the solution being rapidly readjusted to pH 7.0. From this the globulins were slowly precipitated by allowing $(NH_4)_2SO_4$ to diffuse into the solution. This precipitate was again extracted with 0.25 M $(NH_4)_2SO_4$ and the procedure was repeated. Finally, a product was obtained that assayed 115 growth units per mg. which the authors stated represents a concentration of 160-fold.

White and Bonsnes[80] made use of the ultracentrifuge in preparing growth extracts. A 2 per cent NaCl extract of beef anterior lobes was adjusted to pH 5.5, the precipitate discarded, and the supernatant adjusted to pH 4.9. The resulting precipitate was dissolved, adjusted to pH 7.4 and ultracentrifuged for 2 hours at 750 r.p.s. (142,000 g.). The supernatant fluid was removed from any sedimented material and the pH adjusted to 4.9. The precipitate, although still containing recognizable quantities of other pituitary hormones, had a marked growth-promoting activity in hypophysectomized rats.

In TABLE 3 are listed some of the preparations outlined above, together with an estimate of the quantity of material or nitrogen required to produce the unit response in either normal plateaued female or hypophysectomized rats. It will be observed that several groups of investigators have obtained preparations in which approximately 10 to 20 γ of the solid material, containing 1 to 2 γ of nitrogen, when administered daily to hypophysectomized rats would produce a gain of 1 gm. a day. None of these preparations can be considered to represent an isolation of the growth hormone in pure form, since they are either contaminated with other active principles or else still contain inert protein material.

[78] Fevold, H. L., Lee, M. O., Hisaw, F. L., & Cohn, E. J.   Encodrinology 26: 999. 1940
[79] Teel, H. M.   Science 69: 405.   1929.
[80] White, A., & Bonses, R. W.   Unpublished data.

TABLE 3
PREPARATIONS OF GROWTH HORMONE

Investigators	Type of preparation	N₂ content gm. per cent	Plateaued-female rats		Assay — Hypophysectomized rats	
			Solids mg./unit	Nitrogen γ/unit	Solids mg./unit	Nitrogen γ/unit
Evans & Simpson (1933)	1. Alkaline extract	13 18	6 17	1010 2300		
	2. Acetone-dried powder	13 25	5 13	970 2300		
	3. Isoelectric precipitate from (2)					
	4. Supernatant from (3)	12 18	5	580 800		
	5. 20% Na₂SO₄ precipitate	8 17 / 14 15	5 / 5 9	330 790 / 700 1300		
Evans, et al., (1940)	6. Alkaline extract	16 (?)	7.2	1150 (?)		
	7. Cu(OH)₂ extract	16 (?)	0.8	128 (?)		
	8. 'T' fraction (NH₄)₂SO₄ precipitate					
	9. Globulin fraction	16 (?) / 16 (?)	1.3 / 0.8	208 (?) / 128 (?)	0.05 0.10 / 0.012 0.021	7.5 15.0 (?) / 1.9 3.8 (?)
	10. Cysteine-treated globulin fraction	16 (?)	0.5	80	0.012–0.040	1.9–0.4 (?)
Van Dyke & Wallen-Lawrence	11. 20% Na₂SO₄ precipitate and isoelectric precipitation	5 (dry weight)			0.35	17.5 (per 100 gm. rat)
Dingemanse & Freud (1935)	12. Acetone powder				6–10	
	13. Dialysate absorbed on "Norite"	14.3			0.01	1.4
Collip, et al., (1933)	14. Adsorbate on Ca₃(PO₄)₂	16 (?)			0.10	16.0 (?)
Fevold, et al., (1940)	15. pH 8.0 extract of glands	16 (?)			1.3	208 (?)
	16. pH 5.4 solution in 0.25M (NH₄)₂SO₄	16 (?)			0.25	40 (?)
	17. Repeated (NH₄)₂SO₄ precipitation	16 (?)			0.009	1.4 (?)
White & Long (1941)	18. Isoelectric precipitation and ultracentrifugation	14.1			0.025	3.8

METABOLIC CHANGES INDUCED BY ANTERIOR PITUITARY EXTRACTS

A large number of alterations in many phases of metabolism have been reported to follow the injection into normal animals of anterior pituitary extracts of various types. Indeed, so many observations have been reported that it is not an easy matter to present any coherent explanation of the series of events that occur. These difficulties arise by reason of the fact that the anterior pituitary secretes not only hormones that influence the tissues directly but also those that exert their effect by stimulating other endocrine glands.

It may be stated that there are four possible ways in which an anterior pituitary extract may influence the metabolism of the organism:

A. By those hormones whose secretions act directly on the tissues; this effect may be one involving practically all the tissues or may be limited to certain organs. Examples of this type are probably the growth and lactogenic hormones.

B. By the stimulation of other endocrine glands through the specific tropic hormones contained in the extract. Examples of these are the thyrotropic and adrenotropic hormones. Since the effects that follow the action of tropic hormones are due to the particular hormones they liberate, it follows that for an understanding of this particular type of pituitary action we must have information regarding the mode of action of such glands as the thyroid and adrenal cortex. Although much information of this character is available it is by no means complete, so that often considerable doubt exists as to whether a particular effect is attributable to their action.

C. The third possibility is one that does not directly involve either of the two foregoing types of pituitary hormones. It is that the alterations in metabolism produced by an anterior pituitary extract may ultimately involve an alteration in function of another endocrine gland which itself is not directly under the control of the anterior pituitary. The best example of this kind is the behavior of the insulin-secreting cells of the pancreas.

D. Finally, to render the situation still more complicated, the long-continued injection of anterior pituitary extracts may induce a condition in which the animal will neutralize not only the active principles in the extract injected but apparently also the secretion of his own pituitary. Thus, Severinghaus and Thomson[31] have reported that the long-continued injection of such extracts into dogs leads to atrophy of the gonads

[31] Severinghaus, A. E., & Thomson, K. W. Am. Jour. Path. 15: 391. 1939.

and thyroid, and the occurrence of characteristic changes in the animal's pituitary. Consequently, such animals, far from exhibiting the metabolic changes associated with hyperthyroidism, actually showed those associated with hypofunction of this gland.

In view of all these possibilities it is not surprising that what amounts to despair has colored much work in this field. Some investigators have chosen to assume that each newly observed metabolic aberration was due to the operation of a specific metabolic hormone so that it is quite easy to compile a long list of "metabolic" hormones which on my last count had reached a total of 9 and which if added to other known active principles places this organ in a class by itself, for although many steroids have been isolated from such endocrine glands as the adrenal cortex and gonads only a small number have biological activity.

It is my opinion that the number of "metabolic" hormones is much smaller than has been suggested, but before discussing this further it would be well to review briefly the major changes in metabolism that follow the injection of crude anterior pituitary extracts into normal animals.

(1) General metabolism
    (a) Increased basal metabolic rate.
    (b) Growth.
(2) Carbohydrate metabolism
    (a) In fasted animals A.P.E. decreases the blood glucose content.
    (b) In fed animals, particularly those fed carbohydrate, A.P.E. produces hyperglycemia, a depression of the respiratory quotient and an increased accumulation of muscle and liver glycogen (glycostatic action). In the dog the effects are much more pronounced. Glycosuria occurs and if the injections are long continued a permanently diabetic state is established (diabetogenic action).
    (c) A.P.E. produces an increased resistance to the hypoglycemic action of insulin (glycotropic action).
    (d) In the rat A.P.E. brings about an increased insulin content of the pancreas but in the dog there follows a rapid decrease in insulin content.
(3) Protein metabolism
    (a) In fed, fasted or phloridzinized animals a single injection of A.P.E. is followed by a decreased nitrogen excretion in the urine and a decrease in the non-protein nitrogen content of the blood.
    (b) With continued injections of A.P.E. animals exhibit a gain

in weight which is characterized by a greater proportion of water, ash and protein retention and a smaller proportion of fat than are found in pair-fed untreated animals.

(4) Fat metabolism

(a) In fasted animals A.P.E. causes an increased rate of fat catabolism as is shown by an increased content of acetone bodies in the blood and the occurrence of acetonuria (ketogenic action). This also occurs in fed dogs but is not so marked in other species.

(b) There is also an accumulation of liver fat and a decreased fat content of the carcass.

(5) Salt and water metabolism

(a) Diuresis.

(b) A retention of potassium, phosphorus and calcium elements associated with the growth of the tissues and skeleton.

In attempting to interpret these changes in terms of the various hormones and metabolic interrelationships that are involved it is an advantage to consider them in terms of the various ways suggested above in which the anterior pituitary hormones may influence metabolism.

METABOLIC EFFECTS ELICITED BY TROPIC HORMONES

The Thyroid

The increased basal metabolism that follows A.P.E. injection is due at least in part to thyroid stimulation. Certain reservations must be made even for this statement. In fed animals the increase in oxygen consumption occurs soon after the A.P.E. is injected and is not prevented by removal of the thyroid.[46,82,83] The slower increase, occupying several days, which is characteristic of the thyroid hormone itself is abolished by a thyroidectomy prior to the injection of A.P.E.

Hypophysectomized rats have a decreased intestinal absorption rate of glucose[84] and the same was shown to be true in thyroidectomized rats.[85] Russell[86] demonstrated that the defect in hypophysectomized rats was due to thyroid hypofunction by showing that treatment of these animals with thyroxin restored the absorption rate to normal and increased the basal metabolic rate without, however, correcting the other metabolic deficiencies of these animals. Single injections of A.P.E. into normal animals over short experimental periods does not alter the glucose ab-

[82] O'Donovan, D. K., & Collip, J. B. Endocrinology 23: 718. 1938.
[83] Riddle, O., Smith, G. C., Bates, R. W., Moran, C. S., & Lahr, E. L. Endocrinology 20: 1. 1936.
[84] Phillips, R. A., & Robb, P. Am. Jour. Physiol. 109: 82. 1934.
[85] Althausen, T. L., & Stockholm, M. Am. Jour. Physiol. 123: 577. 1938.
[86] Russell, J. A. Am. Jour. Physiol. 122: 547. 1938.

sorption rate[56] since there is not time for the hyperactivity of the thyroid to be exerted.   It is to be anticipated, however, that after longer periods of treatment an increased rate of absorption would be observed.

There are, of course, other effects of thyroid stimulation which will become more manifest the longer the time of treatment with A.P.E.   For example, Fraenkel-Conrat, Simpson and Evans[59] have shown that a marked absolute and relative increase in liver size follows treatment of rats with purified thyrotropic hormone.   It was also demonstrated that an increased liver size follows the injection of purified adrenotropic hormone but growth preparations freed of these two substances bring about a relative decrease in liver size.   Since whole pituitary extracts cause an increase in liver size[15] this work furnishes a good example of the clarification of the problem that will follow the successful separation of the various pituitary factors.

The Adrenal Cortex

The role played by the adrenal cortex in the regulation of metabolism has remained very obscure until recent years.   It is now known that this gland is not only a regulator of the salt and water balance of the body but also through some of its hormones, notably those of the corticosterone type, it exerts an influence on protein and carbohydrate metabolism.   These last effects are as follows: (1) Injection into fasting animals increases to a marked degree the liver glycogen content and to a less degree the blood glucose level, the muscle glycogen remaining unchanged.   (2) Coincident with the absolute increase in the carbohydrate content of the body there is an increase in urine nitrogen.   This increased quantity of protein catabolism is sufficient in magnitude to account for the extra carbohydrate found.[57]   In other words, gluconeogenesis from protein is stimulated by these hormones which in turn implies that under their influence the rate of protein catabolism is increased. This interpretation of the action of these hormones finds support in certain characteristics of the metabolism of adrenalectomized animals which in turn have much in common with those observed to follow hypophysectomy.

When fasted both hypophysectomized and adrenalectomized animals develop hypoglycemia, the former, however, more rapidly and to a greater degree than the latter.   Both have a much reduced liver glycogen content but adrenalectomized animals maintain their muscle glycogen fairly well, but those of hypophysectomized animals are rapidly depleted. This last fact, together with the high rate of carbohydrate utilization of

⁵⁷ Long, C. N. H., Katzin, B., & Fry, E. G.   Endocrinology 26: 309.   1940.

the latter constitutes a very significant difference in the metabolism of the two types of endocrine deficiency and indicates that not all the defects in carbohydrate metabolism after hypophysectomy are to be attributed to adrenal cortical hypofunction.

Both preparations exhibit significant defects when placed under conditions which normally invoke an increased rate of protein catabolism and glyconeogenesis. Among these are exposure to cold, pyrogenic agents, pancreatic diabetes, phloridzin diabetes, exposure to low oxygen pressures and, finally, such a comparatively mild circumstance as fasting. In all these instances not only is the rate of protein catabolism, as measured by the nitrogen excretion, significantly lower than normal but also the rate of gluconeogenesis as measured by the carbohydrate levels of the body or glycosuria. Indeed, the profound hypoglycemia and depletion of carbohydrate stores that occur in fasting hypophysectomized rats may be prevented by the administration of adrenal cortical hormones.

One point of difference in the behavior of the protein metabolism in these two glandular deficiencies should, however, be noted. Immediately after hypophysectomy in rats there is a greatly increased rate of nitrogen excretion, but after adrenalectomy the output is much less than normal.[36] If the hypophysectomized rats are tested some time after the operation, however, an actual reduced nitrogen excretion may be observed, possibly due in part to the ensuing adrenal atrophy and in part to the depletion of, and inability to restore, any depot protein. These observations not only emphasize the complex character of the metabolism after hypophysectomy but also indicate the biphasic character of the endocrine control of the protein metabolism.

Another fact in common to hypophysectomized and adrenalectomized animals is their extreme sensitivity to insulin. Furthermore, the hypoglycemic action of insulin in normal animals can be greatly reduced or abolished by the injection of A.P.E. (glycotropic action). Jensen and Grattan[39] have shown that the injection of cortical extract or adrenal steroids of the corticosterone type will also abolish the convulsive action of insulin in mice. Finally, they were able to show that the same result could be obtained by treatment of the animals with a fairly well purified adrenotropic preparation. Consequently, they suggested that the "glycotropic" hormone of the anterior pituitary was identical with the adrenotropic hormone and that the protective action was due to the liberation of the adrenal cortical steroids. These increased the available carbohydrate of the body by increasing the liver glycogen and this

[36] Fry, E. G., & Long, C. N. H. Unpublished data.
[39] Jensen, H., & Grattan, J. F. Am. Jour. Physiol. **129**: 470 1934-40.

in turn moderated the hypoglycemic action of injected insulin. It might be pointed out, however, than an anti-insulin action of A.P.E. could also be achieved by an agent that inhibited glucose utilization in the tissues, so there is the possibility that two types of "glycotropic" action are invoked by crude A.P.E., although, as will be shown, there is also evidence that certain adrenal steroids inhibit carbohydrate utilization in the peripheral tissues.

One of the most dramatic consequences of the injection of anterior pituitary extracts into fed animals, particularly dogs, is the development of hyperglycemia and glycosuria accompanied by other signs of the diabetic state such as the occurrence of acetonuria. In other species this "diabetogenic" action is less marked but nevertheless can be detected by such procedures as the measurement of the respiratory quotient or glucose tolerance curve; both of which indicate that under the influence of A.P.E. there is a marked depression of the ability of the organism to utilize carbohydrate. The counterpart of the "diabetogenic" action of A.P.E. is the alleviation of a total pancreatic diabetes by hypophysectomy—the Houssay phenomenon. Though it has been maintained that both the "diabetogenic" and Houssay effects were not mediated by other endocrine glands, Long and Lukens[90] were able to show that adrenalectomy also alleviated a total pancreatic diabetes to a comparable degree. In addition, Long, Katzin and Fry[7] found that the diabetes of partially depancreatized rats could be aggravated by adrenal cortical steroids of the corticosterone type. Finally, Ingle[91] has been able to produce profuse glycosuria in a normal rat injected daily with 11-dehydro-17-hydroxy corticosterone.

These experiments not only suggest that a large part of the "diabetogenic" activity of the anterior pituitary is mediated by the adrenal cortex, but that this endocrine gland is capable of stimulating gluconeogenesis in the liver and of inhibiting glucose utilization in the tissues. It might be noted that these two effects have long been regarded as the characteristic disturbances of diabetic metabolism.

ANTERIOR PITUITARY FACTORS ACTING DIRECTLY ON THE TISSUES

If we exclude the possibility that all of the "diabetogenic" action of A.P.E. is exerted directly on the tissues we must still consider what hormones of this gland are to be regarded as exerting their full effects in this manner. Of these the lactogenic and growth-promoting hormones are

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[90] Long, C. N. H., & Lukens, F. D. W. Jour. Exp. Med. **63**: 465. 1936
[91] Ingle, D. J. Endocrinology **29**: 649. 1942.

the only ones that are sufficiently well defined, although even in the case of these hormones it has been shown that the presence of certain other hormones is necessary for their complete action.

The fact that extracts containing the growth hormone reduce the quantity of protein undergoing catabolism implies that the organism must shift to some other foodstuff to replace the calories lost by this reduction in the proportion of protein in the metabolic mixture. One might anticipate, therefore, that the injection of growth-promoting extracts would increase fat catabolism which under suitable conditions would give rise to acetonemia and acetonuria as well as increasing the quantity of the liver fat. It will also be recalled that in the experiments of Lee and Schaffer the treated animals gained more protein and less fat than the controls, indicating again that the burden of supporting the metabolism had been shifted to fat. When to this effect of growth hormone is added the effects of an agent that can inhibit carbohydrate utilization it can well be realized that the readjustment in metabolism produced by A.P.E. greatly increases fat catabolism so that it may be suggested that the ketogenic action of such extracts is not directly related to any pituitary agent that controls fat metabolism exclusively, but is a result of changes induced by those agents that control other phases of metabolism.[92]

It may also be pointed out in connection with the experiments of Lee and Schaffer that the increased gain in protein by the treated rats was not isocalorically equivalent to their increased utilization of fat. The treated group of animals had an excess of 141 gm. of protein equivalent to 578 cal. while they had utilized 119 gm. more fat than the controls which is equivalent to 1095 cal. Whether this difference is due to an increase in the basal metabolic rate of the treated group or whether it it may be taken to imply that there is some more significant relationship between protein synthesis and fat utilization are matters for conjecture. It is evident, however, that such an increased quantity of protein synthesis must require a considerable expenditure of energy and this may well have to be met by the combustion of a substance that is not only available in large quantity but also has a high calorific value. Since we are ignorant of so many phases of protein synthesis the suggestion that fat utilization is intimately coupled with this process may be worth further study.

In fasting animals the injection of A.P.E. also reduced protein catabolism. Since during fasting the amino acids furnish the sole source of glucose, it is noteworthy that such injections produce not hyperglycemia

92 Campbell, J., & Keenan, H. C. Am. Jour. Physiol. 131: 27. 1940, state that the pituitary factor that increases the liver fat of mice is not identical with the ketogenic activity of the extract.

but hypoglycemia in fasting rats.[29]   Evidently the decreased quantity of protein available for conversion into glucose reduces the general level of the blood glucose.   In fasting adrenalectomized rats whose capacity for gluconeogenesis is already impaired, the injection of A.P.E. and the resultant reduction in protein catabolism result in such low glucose levels that severe hypoglycemic shock frequently follows such treatment. Here again it would seem unnecessary to suggest, as some have done, that the hypoglycemia is due to a "pancreatotropic" hormone, but rather to regard this event as another example of readjustment in the metabolic mixture available to the animal.

SECONDARY CHANGES IN METABOLISM FOLLOWING THE INJECTION OF A.P.E.

The Anterior Pituitary and the Insulin Content of the Pancreas

Mention was made above of the claims of some investigators regarding the demonstration of the existence of an anterior pituitary hormone that regulates insulin secretion.   These were based on the observations that the injection of certain types of extract produces hypertrophy of the islands of Langerhans in rats and that under some conditions such extracts would also lower the blood glucose level.   Richardson and Young[93] though unable to confirm the claim that a particular type of extract had these properties, were able to show that crude alkaline extracts of the type that produce glycosuria and permanent diabetes in dogs did cause an islet hypertrophy in rats.   Richardson showed[94] that in the dog rendered permanently diabetic by A.P.E. injections, the islands of Langerhans were more or less destroyed.   Consequently, there exists a marked species difference in the response of these cells to A.P.E. and it is to be noted that this may be correlated with the resistance of the rat to the "diabetogenic" action of the extract.

Best, Campbell and Haist[95] were able to report that in the dog A.P.E. injections immediately and drastically lowered the insulin content of the pancreas, but in contrast Marks and Young[96] found that the same type of extracts increased the insulin content of the rat pancreas.   They also noted[97] that this "pancreatotropic" action was associated with the growth and diabetogenic activity of the extracts.

Further evidence that the influence of the anterior pituitary on the pancreas is an indirect one is furnished by studies on hypophysectomized

[93] Richardson, K. C., & Young, F. G.   Jour. Physiol. 91: 352.   1937.
[94] Richardson, K. C.   Proc. Roy. Soc. London. Series B. 128: 153.   1940.
[95] Best, C. H., Campbell, J., & Haist, R. E.   Jour. Physiol. 97: 200.   1939.
[96] Marks, H. P., & Young, F. G.   Lancet 1: 493.   1940.
[97] Marks, H. P., & Young, F. G.   Lancet 2: 710.   1940.

animals. In the case of all other endocrine glands whose activity is governed by a "tropic" hormone, the removal of the pituitary is followed by atrophy and other evidences of hypofunction. Now it is quite evident that hypophysectomized animals do not exhibit any signs of insulin deficiency but on the contrary many of their reactions suggest an abnormally high ability to utilize carbohydrate. Furthermore, there is no histological evidence to suggest that the islands are not normal in hypophysectomized animals. Indeed, Bakay[98] found that in dogs 2 to 6 months after hypophysectomy the islands of Langerhans were increased both in size and number.

Apart from this morphological evidence Haist[99] has shown that hypophysectomy only slightly reduces the insulin content of the pancreas of rats and, furthermore, that feeding fat causes a decrease as it does in intact rats. The reduced insulin content is restored both in normal and hypophysectomized rats by returning them to a mixed diet. Griffiths[100] has also shown that the injection of A.P.E. into hypophysectomized animals increases the insulin content of the pancreas at the same time that it promotes growth. Moreover, Soong[101] reported similar results. Evidently the capacity of the islet tissue is not only unaffected by hypophysectomy but it is also able to respond in a normal manner to alterations in the demands of the tissues for insulin.

On the other hand, Funk and coworkers[102] in a recent short communication claimed that alkaline extracts of the pituitary can be fractionated into two parts, one of which increases while the other decreases the insulin content of the rat pancreas. The magnitude of the decrease in insulin content that they reported to follow the one fraction is not impressive although, in accord with the work cited above, their "globulin" fraction did increase the insulin content. They also made the surprising claim that highly purified lactogenic hormone will bring about a virtual disappearance of insulin in the rat pancreas. This is surprising not only because prolactin has not been shown to be diabetogenic but also because Fraenkel-Conrat and coworkers[103] reported that this hormone increases the insulin content. Furthermore, purified growth preparations produce a decrease in the insulin content, a result that may be in keeping with the known close association between growth and diabetogenic action. The fact that prolactin is reported to increase the content of insulin in the pancreas is exceedingly interesting, as no other metabolic

[98] **Bakay, L. V.** Pfluger's Archiv. ges. Physiol. **243**: 733. 1940.
[99] **Haist, R. E.** Jour. Physiol. **98**: 419. 1940.
[100] **Griffiths, M.** Jour. Physiol. **100**: 104. 1941.
[101] **Soong, H. Y.** Chin. Jour. Physiol. **15**: 335. 1940.
[102] **Funk, C., Chamelin, I., Wagreich, H., & Harrow, B.** Science **94**: 260. 1941.
[103] **Fraenkel-Conrat, H. L., Herring, V. V., Simpson, M. E., & Evans, H. M.** Am. Jour. Physiol. **135**: 404. 1942.

change (except lactation) has so far been observed to follow the injection of this hormone into mammals.

There are also reports which suggest that the anterior pituitary hormones are not the only ones that may cause alterations in the insulin content of the pancreas. Griffiths, Marks and Young[104] found that, following the implantation of tablets of diethyl stilbestrol, estriol and estradiol into rats, the insulin content of the pancreas was increased; cholestrol, estrone and testosterone were inactive. Funk and co-workers[102] also stated that stilbestrol and estradiol increased the insulin content and that progesterone and testosterone caused a small decrease. It should be noted that stilbestrol produced glycosuria in normal rats[105] though it is stated that the estrogens aggravated the glycosuria of partially depancreatized ferrets.[106] Fraenkel-Conrat and collaborators,[107] however, have recently reported that this effect of estrogens in increasing the insulin content is mediated by the anterior pituitary since they are ineffective in this regard in hypophysectomized rats.

The marked effects of certain adrenal steroids on carbohydrate metabolism is discussed elsewhere. Haist and Best[108] reported that adrenalectomy did not alter the insulin content of rats given a balanced diet and maintained with sodium chloride. The administration of a high fat diet led to a decrease in insulin content but when 3 to 6 cc. of cortical extract was given by mouth to adrenalectomized rats on a balanced diet no change could be detected in the insulin content of the pancreas. But since 11-dehydro 17-hydroxy corticosterone will produce glycosuria in normal rats,[91] it would be well to reserve judgment on the influence of adrenal steroids of this type since the quantities of extract given in these experiments may not have been sufficient to produce detectable effects.

Present evidence would indicate that the production of insulin is not directly dependant on endocrine factors. It seems entirely probable that the demands of the organism expressed through the level of the blood glucose are the factors that regulate insulin production and supply. Circumstances that depress the general level of blood glucose lessen the requirements for insulin but those that result in an elevation of blood glucose stimulate an increased insulin production. A continued stimulation such as is produced by a continued high level of blood glucose appears to bring about, at least in some species, an ultimate failure of the insulin secretory mechanism.

[104] Griffiths, M., Marks, H. P., & Young, F. G.   Nature 147: 359.   1941.
[105] Ingle, D. J.   Endocrinology 29: 838.   1941.
[106] Dolin, G., Joseph, S. J., & Gaunt, E.   Endocrinology 28: 840.   1941.
[107] Fraenkel-Conrat, H. L., Herring, V. V., Simpson, M. E., & Evans, H. M.   Proc. Soc. Exp. Biol. & Med. 48: 333.   1941.
[108] Haist, R. E., & Best, C. H.   Am. Jour. Physiol. 133: 310.   1941.

The apparent importance of the blood glucose level is illustrated by the experiments of Lukens and Dohan. These investigators have previously shown[109] that insulin treatment of cats with a permanent diabetes induced by anterior pituitary extract was followed by morphological and functional recovery of the pancreatic islands. They recently found[110] that a course of phloridzin injections, which markedly lowered the blood glucose of these animals, was also followed by recovery as judged by the subsequent absence of glycosuria and a normal blood glucose level.

The experiments quoted above furnish an excellent example of the manner in which hormones that are concerned with one particular effect on metabolism—in this case the repression of glucose utilization and the stimulation of glucose production—may so alter the character of the metabolism that other endocrine glands may be involved, first by increasing their hormone production and finally, if the initial stimulus is long continued, by an actual decline in function. If the original stimulus is withdrawn, the abnormality in metabolism will be perpetuated by the deficiency in the endocrine that was secondarily involved.

Three ways have been outlined in which the anterior pituitary gland may influence the processes of metabolism and an attempt has been made to present examples of each kind of effect that may be produced as well as the metabolic interrelationships that may be involved. The fourth possibility has not been mentioned, namely, the "anti-hormone" effects that may be produced by long continued injection of A.P.E., since it seems that this can hardly be a physiological process and also it is extremely unlikely that the hormones in a pure form will produce "anti-hormones."

Enough, however, has been said to illustrate the complicated character of the endocrine control of metabolism, but in spite of the fact that emphasis has been placed on the influence of the hormones on metabolism, it should not be forgotten that the ability to utilize or oxidize foodstuffs is an inherent property of the cells and that the endocrine glands only accelerate or inhibit certain phases of these transformations.

Thus, although it has been said in the past that in the absence of insulin the organism is unable to utilize glucose, recent research has shown that the removal of the hypophysis or adrenals restores to some degree the ability to oxidize this carbohydrate. Similarly, thyroidectomy, although reducing the rate of oxidation in cells, does not completely suppress it and hypophysectomy, although greatly retarding protein synthesis,

[109] Lukens, F. D. W., & Dohan, F. C.   Science **92**: 222.   1940.
[110] Lukens, F. D. W., & Dohan, F. C.   Am. Jour. Physiol. **133**: 868.   1941.

does not entirely prevent growth since regeneration of such organs as the liver, although somewhat retarded, still occurs after its removal.

The exact manner in which a hormone diverts the metabolism in one direction or another is still unknown.   It may be that just as certain vitamins have been shown to be parts of enzyme systems concerned with particular chemical transformations, so ultimately a similar role will be assigned to the hormones.